MEMOIRS OF THE SOCIETY FOR ENDOCRINOLOGY

No. 3

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THE TECHNIQUE AND SIGNIFICANCE OF OESTROGEN DETERMINATIONS

Proceedings of a conference held jointly by the Society for Endocrinology and the Endocrinological Section of the Royal Society of Medicine, at the Royal Society of Medicine, London, on 17 February, 1954

Edited on behalf of the Society for Endocrinology by

P. ECKSTEIN & S. ZUCKERMAN

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A NEW METHOD FOR THE DETERMINATION OF OESTROGENS IN URINE AND ITS APPLICATION TO A STUDY OF THE OESTROGEN EXCRETION IN THE MENSTRUAL CYCLE

By J. B. BROWN

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SUMMARY

A convenient chemical method for estimating oestriol, oestrone and oestradiol-17β separately in human urine is described. Evidence is presented for its specificity and accuracy when applied to the urine of men and non-pregnant women. The method was applied to a study of oestrogen excretion during the normal menstrual cycle, and the results obtained are described.

Further research on the biological actions of the oestrogens depends to a considerable extent on the development of suitable assay methods. It was for this reason that, 4½ years ago, *Dr Bauld and I set to work, under Prof. Marrian's supervision in Edinburgh,* to devise an accurate chemical method for estimating oestriol, oestrone and oestradiol-17β in non-pregnancy urine. Because of obvious difficulties, it was decided after some time that it would be wiser for Dr Bauld and myself to work more or less independently towards the same goal. As a result two very different but equally satisfactory methods have been developed. The only major feature common to them both is the new Kober colour method by which the oestrogens are estimated. This colour method, in which the reagents are sulphuric acid and quinol, is a further development by Dr Bauld of a method already described [Brown, 1952] and will be published elsewhere by Dr Bauld [1954]. It is reliable in the presence of urine extracts and is sensitive enough to estimate oestrogens in non-pregnancy urine.

This colour method has been the key to many otherwise baffling problems. Earlier inconsistencies in the behaviour of the oestrogens and inexplicable losses in extraction procedures were found to be due almost entirely to the inadequacy of the colour or fluorescence methods used at the time.

METHOD

The present urinary oestrogen method is summarized in the flow sheet (Fig. 1) and will be published in more detail elsewhere.

Urine is hydrolysed by boiling for 1 hr with 15 vol % of concentrated hydrochloric acid. Rosenmund [1948] and Van Bruggen [1948] have suggested that the partial loss of oestrogens which is said to occur during acid hydrolysis is due to oxidation, and have added reducing agents to prevent this. The efficacy of this treatment could not be confirmed, nor could better hydrolysis conditions be found than those just described.

The hydrolysed urine is extracted with di-ethyl ether.

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The ether soluble acid fraction is then removed from the ether extract. This is usually performed at this stage by washing the ether extract with sodium bicarbonate solution or at a later stage by ether extracting aqueous solutions adjusted to pH 9 to 9 5 [Cohen & Marrian, 1934; Engel, Slaunwhite, Carter & Nathanson, 1950]. At the higher pH the acid fraction is removed more effectively, but some care is required to prevent loss of oestriol. However, it was found that oestriol is easily extracted with ether from aqueous solutions at a pH as high as 10.5, provided the aqueous solutions are saturated with sodium carbonate or bicarbonate at that pH. In fact, the partition coefficient of oestriol between ether and concentrated carbonate solution of pH 10.5 is the same as between ether and saturated sodium bicarbonate. Concentrated carbonate solution of pH 10.5,

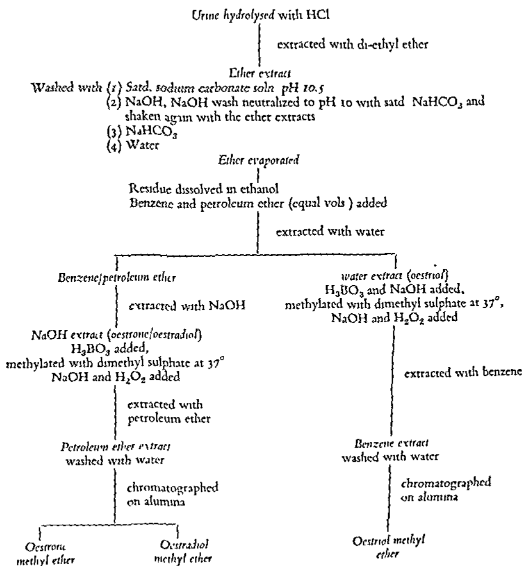


Fig 1 Flow sheet

which is much more effective than sodium bicarbonate, can therefore be used for washing the acid fraction from ether extracts without any additional loss of oestriol.

Urine extracts contain substances, presumably polyhydric phenols, which oxidize to coloured products when dissolved in alkali and are not then re-extractable with ether at $pH > 7$. The apparently involved alkali washes shown in the flow sheet are for the purpose of removing these substances.

The acids are removed by washing with concentrated carbonate solution of pH 10.5. The ether extract is then shaken with sodium hydroxide The polyhydric phenols oxidize and, together with some of the oestriol, are extracted into the sodium hydroxide layer. The sodium hydroxide is then partly neutralized by adding saturated sodium bicarbonate solution The aqueous layer is now a concentrated carbonate solution of pH 10, and shaking again with the ether layer extracts the oestriol back into the ether. The aqueous layer is then discarded, and the ether is washed with saturated sodium bicarbonate to neutralize any alkali still present. This is necessary to prevent loss of oestriol in the subsequent water wash. The ether is then washed with a small amount of water to remove the bicarbonate.

The ether is evaporated and the residue dissolved in ethanol.

The phenolic fraction is usually separated from the neutral fraction, and oestriol from oestrone and oestradiol, by partitions between benzene and water, and benzene and sodium hydroxide. However, difficulty was experienced in getting quantitative recoveries of oestriol and oestrone with these solvents, and it was concluded that their partition coefficients do not favour sufficiently the aqueous phases It is easy in this case to alter the partition coefficients in a favourable manner by diluting the benzene with petroleum ether, in which the oestrogens are relatively insoluble. The mixture of equal volumes of benzene and petroleum ether was found to be very satisfactory. Using this as the organic phase instead of benzene alone, better recoveries of oestriol and oestrone are obtained with fewer extractions and without the formation of emulsions.

Sodium hydroxide is added to the oestriol extract in preparation for the next step

There was reason to believe that much of the material in the oestrogen fractions at this stage is not truly phenolic, and the next step was designed to remove this non-phenolic material. The principle used is phase change through chemical modification of the phenol group. After a few preliminary experiments it was found that methylation is a very convenient method for achieving this, especially as the oestrogen methyl ethers can be estimated directly with the Kober colour method The complete phase change obtained by methylation is shown by the fact that whereas oestriol is extracted from benzene with water, oestriol methyl ether is extracted from sodium hydroxide with benzene; oestrone and oestradiol are extracted from benzene with sodium hydroxide, while their methyl ethers are extracted from sodium hydroxide with petroleum ether.

The oestrogen fractions, already in alkaline solution, are conveniently methylated with dimethyl sulphate, an effective methylating agent under these conditions. The usual methods of methylating phenols with dimethyl sulphate are, however, unsatisfactory when applied to these oestrogen extracts In strongly alkaline solutions, methylation is

slow compared to the rate at which the dimethyl sulphate is destroyed by hydrolysis. It was then found that this destruction is considerably retarded without seriously decreasing the rate of methylation, by methylating in borate buffer solutions of pH between 10 and 11.5. The reaction takes place slowly at room temperature but is conveniently accelerated at 37°C. At this temperature the reaction is complete in 30 min.

The methylation mixture is then made strongly alkaline to emphasize the alkali insolubility of the true methoxy phenols, and hydrogen peroxide is added to destroy certain substances which form a portion of the urinary contamination.

Oestriol methyl ether is then extracted with benzene and the oestrone and oestradiol methyl ethers are extracted with petroleum ether. In each case the partition completely favours the organic phases. The considerable purification of oestrogen extracts obtained by this methylation and phase change procedure is shown by the fact that the benzene and petroleum ether extracts are completely colourless and all the visible pigments are discarded, apparently unchanged, with the aqueous layers.

The oestrogen methyl ether fractions are then further purified, and oestrone and oestradiol are separated from one another by chromatography on alumina columns. Stimmel [1946] was the first to apply adsorption chromatography on alumina columns to the further purification and partition of the oestrogen fractions. However, considerable losses of oestriol occurred during his procedure, and these could not be corrected without considerably increasing the amount of interfering material in the oestriol fraction. The method therefore has not been favourably received. This objection, however, does not apply to oestrogen methyl ethers, for, being less polar, they are more suited to adsorption chromatography procedures.

Adsorption chromatography used as a quantitative procedure has been criticized and condemned by many workers [see Engel, 1950]. In spite of this, the present technique, developed in less than a month, has been in routine use for more than 3 years without giving trouble. It seems to be completely reliable, provided the necessary precautions are taken and all factors are rigidly standardized.

Mixtures of oestrone and oestradiol methyl ethers are resolved with difficulty on columns of active alumina, but are easily resolved on columns of less active alumina. A suitable activity was obtained by adding 9-10% of water to an alumina with an original activity equivalent to Brochman No. II. The activity of this hydrated alumina is practically unaffected by small changes in water content and solvents used with it should be saturated with water. Both these factors contribute to a robust chromatography procedure.

Mutual displacement of adsorbed substances on the columns is avoided by using dilute extracts, already considerably purified and containing only small concentrations of oestrogen methyl ethers.

The chromatography procedure is a compromise between resolution of urine fractions on the one hand and a reproducible, quantitative method with adequate safety margins on the other.

Columns are prepared with 2 g of alumina. The oestrone–oestradiol fraction in petroleum ether is adsorbed on to the column. The column is then eluted with a mixture

of benzene and petroleum ether to remove the pre-oestrone fraction. Oestrone methyl ether is then eluted with a stronger eluant, and elution is continued with the same eluant to remove the inter oestrone-oestradiol fraction, and the oestradiol methyl ether is then eluted with benzene. By using eluants of increasing strengths, fractions are obtained in volumes small enough to be evaporated directly from the colour tubes.

The oestriol fraction in benzene is similarly adsorbed on another column; a pre-oestriol fraction, which contains the only pigment band seen during chromatography, is eluted with a dilute solution of ethanol in benzene and the oestriol methyl ether with a more concentrated solution of ethanol in benzene.

The methylated oestrone, oestradiol and oestriol fractions are evaporated with quinol

colours in the Kober reaction and these are corrected for spectrophotometrically by the method of Allen [1950]. Colour densities are measured in a spectrophotometer at the wavelength 517 mμ, the absorption maximum for the Kober red colour, and at two

One person can conveniently do four complete estimations from hydrolysis of urine to colorimetry in 2 days This allows time for preparing reagents and washing glassware and can easily be doubled for limited periods.

Specificity

The criterion for validity of the spectrophotometric correction is that the wavelength absorption curves of the interfering yellow-brown colours should be linear between the

sorption maxi-

To determine

whether the wavelength absorption curves of the yellow-brown component are in fact linear in this region, it was necessary to apply the method to urines in which low or zero oestrogen levels might be expected. Table 1 shows the results obtained in a series of urines from men, postmenopausal women, children, and patients suffering from Simmond's disease. Deviation from linearity is expressed in terms of micrograms of apparent oestrogen excreted in 24 hr Completely zero levels, implying linearity, were rarely found. The results however are not inconsistent with those which might be expected if these urines contained small amounts of oestrogens The amount of interfering yellow-brown colour produced with these urine extracts is a considerable proportion of the total colour, and it is necessary to rely over much on the spectrophotometric method to correct for this. For instance, in the case of the male urines shown, the density of the yellow-brown component, as calculated by the correction formula, is about 9/10ths of the total in the case of oestriol, 2/3rds in the case of oestrone, and 14/15ths in the case of oestradiol. In the light of this contamination, the significance of the small apparent

oestrogen content of these urines is somewhat doubtful, especially in the case of oestriol and oestradiol.

Table 1 *Values found in expected low titre urines (μg/24 hr)*

	No of cases		Range	Mean
Males	28	oestriol	0.8–7.5	3.2
		oestrone	3.4–8.2	5.7
		oestradiol	0.0–2.4	1.5
Post-menopausal women	9	oestriol	0.6–6.8	3.0
		oestrone	0.8–7.1	2.7
		oestradiol	0.0–2.3	0.3
Children aged <8 years	4	oestriol	0.3–2.4	1.0
		oestrone	0.2–1.0	0.5
		oestradiol	0.0–0.2	0.1
Simmond's disease	4	oestriol	1.2–4.6	2.4
		oestrone	0.0–3.6	1.7
		oestradiol	0.0–1.5	0.5

There is a certain amount of evidence, however, that these values might represent true oestrogen levels. The substances being measured behave chromatographically as oestrogen methyl ethers. The Kober reaction, giving a colour with an absorption maximum at 517 mμ is known to be remarkably specific for the natural oestrogens. The wavelength absorption curves of colours produced with these urine fractions are completely consistent with the presence of two components, one with an absorption maximum at 517 mμ and the other with a linear wavelength absorption curve in this region of the spectrum (Fig. 2).

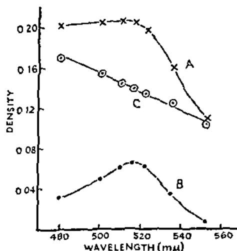


Fig 2 Wavelength absorption curves of colours produced in the Kober reaction A, oestrone fraction from ¼ of a 24 hr male urine, calculated by correction formula to contain 1.4 μg of oestrone methyl ether, B, pure oestrone methyl ether 1.4 μg, C, background material calculated from A minus B

Further support is afforded by results obtained biologically for the excretion of oestrogens in male urine. Although these biological methods are liable to considerable errors, the results obtained are of the same order as those obtained with the chemical method. For instance, Gallagher, Kenyon, Peterson, Dorfman & Koch [1937] found in men's urine an average oestrogen concentration equivalent to the excretion of 10 μg of oestrone a day, results which compare favourably with those shown here.

Accuracy

The accuracy of the method was tested by a series of recovery experiments in which known amounts of oestriol, oestrone and oestradiol-17β were added to portions of acid-hydrolysed 24 hr male urines. Blank determinations were made at the same time on the same urine. Results are shown in Table 2 and are expressed in terms of mean values and

Table 2. *Recoveries from acid-hydrolysed male urine.*
(*Results shown as mean % ± S.D. and corrected for endogenous blank values*)

	μg added per 24 hr urine 4-7	25-35	36-60
Oestriol	88 ± 10.8 (12)	85 ± 3.8 (11)	83 ± 3.0 (12)
Oestrone	87 ± 11.7 (11)	84 ± 5.2 (12)	82 ± 6.1 (10)
Oestradiol	80 ± 5.5 (12)	91 ± 7.0 (11)	86 ± 6.6 (11)

standard deviations. The amounts of oestrogens added per 24 hr urine specimen are shown, and results are the percentage of this recovered after subtracting the urine blank values. Recoveries are between 80 and 90%, even at levels corresponding to 4 μg per day, which was the lowest investigated. The standard deviations, however, increase as the amount of added oestrogen is decreased. This is not surprising, for at the concentrations being measured the oestrogen colour densities are so low that considerable instrumental errors arise during their measurement. Also, at these levels the apparent oestrogen content of the blank forms a considerable proportion of the whole, and errors in measuring this contribute to the total error.

These satisfactory recovery results show that the method, whatever it measures, is at least reliable for measuring differences in oestrogen concentrations in the same urine.

RESULTS

Oestrogen excretion during the menstrual cycle

The method was then applied to a study of oestrogen excretion during normal menstrual cycles in women. Urines were collected daily throughout twelve menstrual cycles from ten apparently normal women whose ages ranged from 17 to 40 years. Pregnancy intervened in three of the cycles studied. Basal temperatures were taken daily throughout

each cycle in order to compare oestrogen levels with times of expected ovulation Fig 3 shows the results obtained from a typical cycle, and illustrates a number of features common to all the cycles studied.

There are two peaks of oestrogen excretion. The sharper and usually the higher peak occurs about the 13th day (or more constantly, 15 days before the onset of menstruation) —just about the time of the temperature rise, and is presumably related to ovulation.

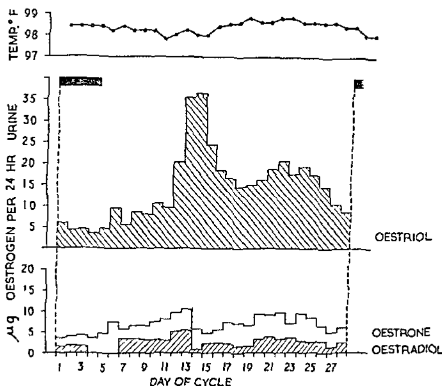


Fig 3 Excretion of oestriol, oestrone and oestradiol during a menstrual cycle Days of menstrual bleeding and basal temperature curve are shown Subject 1, age 36, para 3

This is followed by a sudden drop in oestrogen excretion, which then rises to a second peak at about the 21st day of the cycle. The levels then fall before the onset of menstruation and reach their lowest about 2 days after the onset of menstruation These trends are shown by all three oestrogen fractions

The finding of two peaks of oestrogen excretion during the menstrual cycle is not new and has been recorded by many workers in the past, using bioassay methods. For instance, Smith, Smith & Pincus [1938] found two peaks of oestrogen excretion, one about the 10th day and the other about the 18th day of a 24-day cycle. The oestrogen levels at the first peak (calculated from their data and assuming an oestrone : oestradiol ratio of 2 . 1) were oestriol, 30 µg, oestrone plus oestradiol, 20 µg, which are very similar to those found here

The relative amounts of oestriol, oestrone and oestradiol found in these menstru

cycle urines are interesting. The amount of oestradiol is usually about half that of the oestrone in the same urine specimen, and daily changes in the concentration of the one are reflected in parallel daily changes in the concentration of the other. The relative amounts of oestriol and oestrone are variable and the daily changes in the oestriol levels are not reflected directly in the changes in the oestrone levels but tend to lag a day behind them. This is most noticeable during the first oestrogen rise and at the first peak, and is shown clearly in Fig. 3 Here, the oestrone and oestradiol peaks occur on the 13th day, but the corresponding oestriol peak does not occur until the 14th day.

These relationships between the amounts of oestriol, oestrone and oestradiol are also found following the single intramuscular injection of oestradiol into postmenopausal women. Fig. 4 shows the results obtained from one such experiment. The peak excretion of oestrone and oestradiol occurs on the 1st day following the injection, and the oestradiol levels are about half the oestrone levels. The oestriol excretion lags behind that of the oestrone and oestradiol and reaches its peak on the 2nd day following the injection This lag phenomenon was also observed under similar conditions by Clayton [1949].

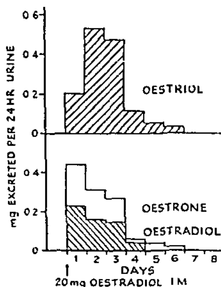


Fig 4 Excretion of oestriol, oestrone and oestradiol following a single intramuscular injection (I M) of oestradiol-17β (20 mg) Total oestrogen recovered was 16% of that injected

The results obtained from menstrual cycles at about the time of the first peak are therefore completely consistent with those expected following the continuous injection of oestradiol, the amounts of which vary from day to day in a cyclic manner

The evidence therefore suggests that this chemical method affords a measure of the excretion of oestriol, oestrone and oestradiol in the urine of men and non-pregnant women.

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DISCUSSION

Miss Stern· In your calibration graph you could measure down to 2 μg of oestradiol, and yet in the actual analysis, in the postmenopausal woman for instance, you measured down to 0 2 μg.

Dr Brown· Yes, but I agree that the Kober method is becoming unreliable at that level, so that we do not place too much importance on very low values, say below 10 μg

Miss Stern· But the actual analysis was on a 24-hr specimen ?

Dr Brown: A fifth of a 24-hr sample.

Miss Stern. You added your oestrogens for recovery after acid hydrolysis Do you think it possible that during hydrolysis there could be some destruction? We seem to have some evidence of that

Dr Brown It is generally accepted that destruction is liable to occur during hydrolysis. The recovery experiments were not designed to check that, but just to see how our extraction procedures were working

Dr Bishop. Has Dr Brown any suggestion why there should be this difference in oestriol and oestrone-oestradiol excretion in nulliparous and parous women?

Dr Brown We would like to know the answer to that ourselves.

Mr Williams Have you done any determinations on urine from hysterectomized women?

Dr Brown We haven't many figures on hysterectomized women, and those we have are on post-menopausal cases

Dr Diczfalusy It may interest you that we have checked the partition data of Dr Brown on the methylated oestrogens, using the Craig countercurrent distribution, and our data so far agree completely.

Dr Hannelore Braunsberg Have you any evidence that quantitative methylation occurs in the presence of impurities? You have added the oestrogens at stage 2 and recovered them again in the presence of urinary residues?

Dr Brown Yes, we have checked that.

SOURCES OF ERROR IN THE CHEMICAL DETERMINATION OF URINARY OESTROGENS

By W. S. BAULD

From the Department of Biochemistry, University of Edinburgh

The investigations reported here were conducted simultaneously with, but independently of, those just reported by Dr Brown. As the method finally developed will be described in detail elsewhere [Bauld & Marrian, 1954], the present discussion will be confined mainly to the major difficulties which arose and the steps taken to overcome them.

METHODS

The original method (shown in outline in Fig. 1) was based on earlier investigations by Clayton [1949]. The new developments are marked in the figure and are as follows:

*The oxidation of phenolic contaminants to more acidic products by shaking an alkaline solution in the presence of air.

**Extraction with ether of the oestriol fraction in N-NaOH to remove neutral and nitrogenous contaminants.

***The use of partition chromatography for the separation and purification of oestrone and oestradiol-17β, and for the final purification of oestriol.

****Chromatographic separation of neutrals and the lipophilic oestrogens.

The difficulties encountered in the application of the method will be discussed under three headings.

Errors in colorimetric assay.

Loss during partition.

Destruction of oestrogens during the procedure.

Errors in colorimetric assay

The introduction by Brown [1952 *a*] of the quinol-aqueous H_2SO_4 reagents was a distinct advance in producing a convenient method of oestrogen determination of much greater reliability than earlier modifications of the Kober [1931] reaction [e g. Venning, Evelyn, Harkness & Browne, 1937]. However, when the method in its original form was extended to the estimation of oestrogens in the presence of urinary and solvent residues, certain forms of intereference with colour development were found. These findings are reported in detail elsewhere [Bauld, 1954*a*] and will be summarized only very briefly here

It was found that for oestriol to form quantitatively the yellow precursor of the final pink colour there had to be an effective reductant present in the first stage of the reaction For the determination of pure oestrogen this requirement is met by the sulphonated quinol of Brown's reagents This weak reducing agent is, however, inadequate in the

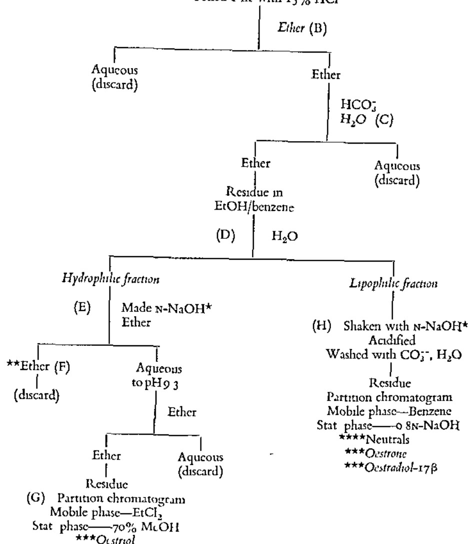


Fig 1 Outline of the basic method for extraction and purification of oestriol, oestrone and oestradiol-17β from urine

presence of oxidants arising from solvent or urinary residues. In a typical experiment 96% of oestriol was recovered on extraction from 1 3 N-H_2SO_4 when the reagent for the colour reaction contained 1.4% (w/v) of free quinol, whereas only 65% was recovered when the free quinol in the reagent was 0 3% (w/v) The difficulty was overcome by the addition of quinol to the reagent immediately before use.

Moreover, for all the oestrogens the second stage of the reaction, the conversion of yellow to pink, appears to be a graded oxidation: with insufficient oxidation, there is incomplete conversion and with excessive oxidation, fading of the pink occurs In the presence of various oxidizing and reducing contaminants it is therefore essential to have conditions of maximum stability for this conversion. Since changes in the intensity of

the pink occur in dilute H_2SO_4 even at room temperature, the dilution with water immediately prior to colorimetry, as required in the original reaction [Brown, 1952 *a*], was omitted. In the second stage, the important factors involved were shown to be: H_2SO_4 concentration, time of heating, and composition of the reagent. Their effects are shown in Fig. 2. In each experiment, eleven samples of oestradiol-17β (25 μg) were heated at 100°C for 20 min with 4 ml. of 2% (w/v) quinol in 60% (v/v) H_2SO_4, cooled, diluted with 3.5 ml. of aqueous H_2SO_4, and reheated. Optical densities obtained with the green (Ilford 604) and violet (Ilford 601) filters are plotted against time of reheating. With Type B H_2SO_4 the conversion of yellow to pink (as evidenced by alteration in the optical densities obtained with both filters) at a second stage concentration of 60% was incomplete even with prolonged heating. When the concentration was dropped to 46%, the conversion to pink was markedly facilitated, but the colour was now susceptible to fading. Moreover, a marked difference was found between the different types of analytical reagent H_2SO_4, both in ease of conversion and in intensity of colour. That Type A H_2SO_4 is still not optimal is shown by the increased density obtained by the addition of Cu^{++} (280 μg) in the second stage of the reaction.

These results and similar findings with the other oestrogens led to minor but important changes in the original reagents and reaction described by Brown [1952 *a*] We are now convinced of the accuracy and reliability of the modified colour reaction in the presence of contaminants from solvents and urine.

Since the development of fluorescence in H_2SO_4 merely represents the first stage of

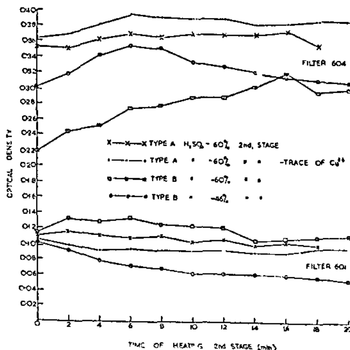


Fig 2 Effect of variation in conditions of the second stage on the conversion of yellow to pink

the Kober reaction, the considerations briefly discussed above will apply at least in part to fluorimetric methods of estimation of oestrogen. Brown [1952 *b*] stressed the necessity for a reducing agent in the development of fluorescence by oestriol. Bates [1952] contested this point, but used oestrone as his example. Most methods meet the requirement by the use of ethanol as a solvent. Veldhuis [1953] found a fifteen-fold increase in the fluorescence of oestriol when ethanol was included in the H_2SO_4 reagent. Conversion of the fluorescent compound to the pink derivative—an event which in view of the low concentrations of oestrogen used for fluorimetry would be demonstrable only by decreased fluorescence—is one of the possible forms of 'quenching'. This may explain why batches of H_2SO_4 from different suppliers vary in their ability to produce fluorescence of oestrogen [see Engel, Slaunwhite, Carter & Nathanson, 1950]. The instability of fluorescence methods in the presence of contaminants from solvents, urine or blood was recognized by Finkelstein [1952] and Veldhuis [1953], who correct for this form of 'quenching' by measuring the fluorescence of a known amount of oestrogen in the presence and absence of an aliquot of their final extract. If fluorescence methods are to have a wide application their stability must be increased

Loss during partition

The variable factors affecting the elution of oestrogens from the partition chromatograms of the final method will be reported in detail elsewhere [Bauld, 1954 *b*]. Two of the more important causes of variation are shown in Fig 3. As the partition coefficients of oestrone and oestradiol-17β between aqueous alkali and benzene depend upon the temperature at which the solvents are equilibrated, this factor becomes important in determining the elution pattern. The variation with percolation rate was shown to be

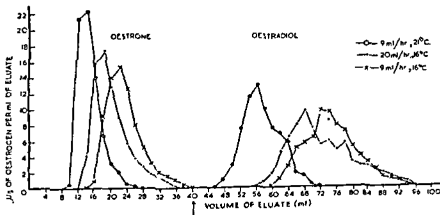


Fig 3 Effect of variations in percolation rate of solvent, and in temperature on elution pattern of oestrone and oestradiol-17β

All columns were 1 × 12 cm NaOH (0 8 N) and benzene were equilibrated at the temperatures shown, the aqueous (stationary) phase was mixed with celite 535 in the proportions of *8 ml per 10 g of celite* Solutes (150 μg) were applied in organic (mobile) phase in 3 × 1 ml washes, and collection of the eluate was begun after the third wash entered the chromatogram The mobile phase was changed at 40 ml to $EtCl_2$-benzene 3/1

dependent upon the alteration in cross-sectional areas of the mobile and stationary phases resulting from the variation in the firmness with which the chromatograms were packed. The tendency to trailing elution boundaries seen in the figure is in accord with the non-linear partition isotherm of oestrogens in this system. In the final method the chromatograms are run in a room controlled at 18 ± 0.5°C; and the columns are packed sufficiently firmly to give a percolation rate of 10 ml /hr with gravity flow.

'Destruction' of oestrogens during the extraction procedure

Because of possible instability of the colour reaction it is difficult to distinguish 'destruction', in the sense of modification of the oestrogen molecule to make it non-chromogenic in the Kober reaction, from apparent loss of oestrogen due to interference with colour development. In the ensuing discussion, 'destruction' will be applied only to losses (in terms of colorimetric estimation) preventable by changes in the procedure prior to colorimetry. Such losses were of four kinds.

1 *'Destruction' of oestriol on extraction from dilute HCl* When the reliability of the projected method was tested by recovery of oestrogens from dilute HCl, satisfactory results were obtained with oestrone and oestradiol-17β, but recoveries of only $64 \pm 11\%$ were obtained in twenty-seven experiments with 25 μg of oestriol added to 575 ml of 1.3 N-HCl. Investigation showed that the principal loss occurred during the first stage of the purification procedure extraction with ether, washing with bicarbonate and water, and distillation of the extract. Yields at this stage were not improved when various other methods of ether purification were substituted for the routine one of shaking with silver oxide [Werner, 1933], or when distillation in an atmosphere of nitrogen, or the addition of ascorbic or 1-amino-2-naphthol-4-sulphonic acids were employed.

It was known [Rosenmund, 1948] that Fe^{+++} caused an appreciable destruction of oestrogens on boiling in dilute HCl It was found that complete destruction of oestriol in dilute solution occurred on prolonged standing at room temperature with 0 1 mg Fe^{+++} per 100 ml. of 1 3N-HCl. Even with immediate extraction there were losses up to 50% This metal-catalysed destruction was prevented, however, by ascorbic acid (1 part in 5000) Moreover, analytical grade reagents were used throughout this investigation, and no traces of Fe^{+++} could be demonstrated in the extracts. It was therefore considered unlikely that this type of destruction was the cause of the low yields on extraction of oestriol from dilute acid.

Oestriol was recovered quantitatively in the first stage of the purification procedure (extraction with ether, washing with bicarbonate and water, distillation of the ether) when quinol (1 part in 5000) was added to the 1.3N-HCl at the start. This antioxidant, unlike ascorbic acid and 1-amino-2-naphthol-4-sulphonic acid, is not completely removed from ether by the bicarbonate washes. Its effectiveness therefore suggested that the 'destruction' took place during distillation of the solvent.

In the absence of quinol, quantitative recoveries from this first stage were found when the dilute HCl was neutralized before extraction, or when oestriol was extracted from water or 1.3N-H_2SO_4 This suggested that HCl was not being completely removed from the ether by the bicarbonate washes and was 'destroying' oestriol during the dis-

the Kober reaction, the considerations briefly discussed above will apply at least in part to fluorimetric methods of estimation of oestrogen. Brown [1952 *b*] stressed the necessity for a reducing agent in the development of fluorescence by oestriol. Bates [1952] contested this point, but used oestrone as his example. Most methods meet the requirement by the use of ethanol as a solvent. Veldhuis [1953] found a fifteen-fold increase in the *fluorescence of oestriol when ethanol was included in the H_2SO_4 reagent* *Conversion of* the fluorescent compound to the pink derivative—an event which in view of the low concentrations of oestrogen used for fluorimetry would be demonstrable only by decreased fluorescence—is one of the possible forms of 'quenching'. This may explain why batches of H_2SO_4 from different suppliers vary in their ability to produce fluorescence of oestrogen [see Engel, Slaunwhite, Carter & Nathanson, 1950]. The instability of fluorescence methods in the presence of contaminants from solvents, urine or blood was recognized by Finkelstein [1952] and Veldhuis [1953], who correct for this form of 'quenching' by measuring the fluorescence of a known amount of oestrogen in the presence and absence of an aliquot of their final extract. If fluorescence methods are to have a wide application their stability must be increased.

Loss during partition

The variable factors affecting the elution of oestrogens from the partition chromatograms of the final method will be reported in detail elsewhere [Bauld, 1954 *b*]. Two of the more important causes of variation are shown in Fig. 3. As the partition coefficients of oestrone and oestradiol-17β between aqueous alkali and benzene depend upon the temperature at which the solvents are equilibrated, this factor becomes important in determining the elution pattern The variation with percolation rate was shown to be

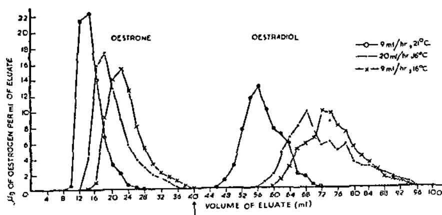


Fig 3 Effect of variations in percolation rate of solvent, and in temperature, on elution pattern of oestrone and oestradiol-

shaken with a small amount of N-NaOH, and the mixture was then shaken with bicarbonate to lower the pH of the aqueous phase to 8.5 before discarding the combined wash. This resulted in an appreciable purification of the final extracts. Ethanolic solutions of the separate fractions of male urine, obtained with this modification of the method, were evaporated to dryness by heating in a stream of air with and without added oestrogens, and the colour reaction was carried out. The results for oestriol are shown in Fig. 4 and Table 1.

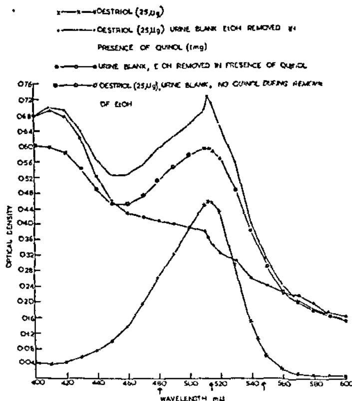


Fig 4 Absorption spectra of colours produced in modified quinol-aqueous sulphuric acid reaction (7 5 ml.) by pure oestriol and oestriol heated with the oestriol fraction of male urine

The optical density at the peak of the oestrogen absorption was considerably lower for oestriol in the presence of urinary residues, whether a correction for the non-pink component was applied or not. The discrepancy was significantly less when the quinol required in the first stage of the colour reaction was added to the ethanolic solution before removal of the solvent. It would seem that heating oestriol with urinary residues causes a 'destruction' which is largely preventable by quinol

In an effort to determine which group of compounds was causing this 'destruction', the alkalization wash of the original ether extract was acidified, extracted with ether, washed with bicarbonate and water, and the solvent removed by distillation The residue,

tillation of the solvent. With the introduction of the strong alkalization of the ether extract (discussed later) no loss on extraction from HCl occurred. Since hydrolysis with this acid gives less contaminated urinary extracts than does equimolar H_2SO_4, HCl has been retained in the final procedure.

2. *'Destruction' by heating with solvent residues.* Other investigators have reported the destruction of oestrogen when an ethanolic solution is heated in a stream of air [see Haenni, 1950; Bitman & Sykes, 1953]. This finding was confirmed with certain batches of ethanol purified by azeotropic distillation with benzene and with ethanol containing appreciable quantities of acetaldehyde. The loss occurred even when the ethanol was removed by heating in an atmosphere of nitrogen under diminished pressure. However, it was found that ethanol, purified during manufacture by simple distillation only and rendered acetaldehyde-free by a two-stage treatment with m-phenylenediamine, could be evaporated from oestrogens without causing any destruction. The effect of traces of aldehyde was enhanced by ether residues. Routinely, therefore, aldehyde-free ethanol is used for the transfer of oestrogens to tubes for colorimetry, and as an additional precaution the quinol required in the first stage of the colour reaction is added. With these precautions no loss of oestrogens occurs when the ethanol is removed by heating in a stream of air.

3. *'Destruction' by heating with urinary residues.* When the method was applied to the recovery of oestrogens added to acid-hydrolysed male urine, additional purification stages were found desirable. The major group of brown contaminants arose from colourless precursors during the extraction procedure, the change occurring whenever the phenolic residues contacted alkali. Thus, in spite of shaking the benzene solution containing oestrone and oestradiol-17β with alkali, and subsequently washing it with carbonate (Step H, Fig 1), a brown band was always left on the chromatogram after elution of the neutrals with benzene. Washing the ether extracts with sulphite or bisulphite effected a purification at this particular stage, but did not result in any appreciable decrease in the contamination of the final fractions The substances concerned appeared to be so susceptible to oxidation to brown derivatives that purification was best achieved by removal rather than by reduction The easiest approach to this was an extension of the procedure for shaking in alkaline solution, followed by washing with weak alkali. Accordingly, the initial ether extract was washed once with bicarbonate and

Table 1 *Corrected optical density (E_{corr}) of colour produced in the modified quinol-aqueous sulphuric acid reaction (7.5 ml vol) by pure oestriol and oestriol heated in ethanol with the oestriol fraction of male urine*

		E_{corr}	% Loss
1	Oestriol (25 µg)	0.269	—
2	Oestriol (25 µg), urine blank, EtOH removed in presence of quinol (1 mg)	0 251	17
3	Urine blank, EtOH removed in presence of quinol	0 034	—
4	Oestriol (25 µg), urine blank, no quinol during removal of EtOH	0 179	38
5.	Urine blank, no quinol during removal of EtOH	0 022	—

buffer of pH 10.5 prior to the alkalization a significant improvement in recovery resulted The use of a 10.5 buffer was introduced by Brown, as has already been described. A 10.5 wash after alkalization did not improve the recoveries It thus appeared that the effectiveness of this wash lay in the fact that it removed substances from the urinary extracts which, if present during the shaking with strong alkali, facilitated the oxidation of oestradiol-17β.

Expt. 3 demonstrated that the causes of 'destruction' were not completely removed because at lower levels of oestrogen the recoveries dropped to 51 ± 8.2%. Preliminary experiments established that some of the loss was due to alkalization of the benzene solution (Stage H, Fig. 1). Elimination of this stage of the purification gave recoveries of 85 ± 5.8% when 10 μg of oestradiol-17β were added to ¼ of a 24 hr specimen of hydrolysed male urine.

As shown by Expts. 5 and 6, however, the results became unsatisfactory as the amount of added oestrogen was decreased. There was a marked variation from urine to urine. It was thought possible that this destruction of minute amounts of oestradiol-17β was occurring during the removal of ethanol after transfer to tubes for colorimetry. This effect at the higher levels of oestrogens has already been discussed; it was shown to be largely overcome by the presence of quinol. Addition of 1 μg of oestradiol-17β to this fraction of the urinary extract just before transfer for colorimetry resulted in losses of 10–20% with certain urines even in the presence of quinol, the losses being greater when the background colour in the fractions was high. Further purification was therefore required.

Saponification of the residue from the chromatogram eluate by refluxing for 30 min in N-NaOH gave a fraction (obtained by benzene extraction of the acidified aqueous phase) which was considerably cleaner and did not destroy oestradiol-17β when heated with it in ethanol. Possibly the harmful agents are esters of phenolic acids. Expt. 7 showed that, with this final modification, oestradiol-17β (1 μg), when added to ¼ aliquot of hydrolysed male urine, was recovered to the extent of 84 ± 6.9%.

The outline of the final method is shown in Fig. 5 The factors affecting the recovery of oestradiol-17β also apply to oestrone but to a lesser extent. Oestrone was found to be the most stable of the three oestrogens, and with it no saponification was necessary.

Table 3 *Recovery of oestriol and oestrone added to acid-hydrolysed male urine*

Oestrogen added to ¼ of 24 hr specimen of acid-hydrolysed male urine	No of expts	% Recovery Mean ± S D
Oestriol 10 μg	12	83 ± 4 7
2	12	87 ± 7 5
1	12	81 ± 10 5
Oestrone 10 μg	16	87 ± 4 4
2	16	92 ± 4 1
1	13	90 ± 7 0

N B All recoveries are corrected for endogenous oestrogen content

in amounts corresponding to 1/50 of the 24-hr urine volume, caused a 'destruction' of 25-26% of oestriol (10 μg) when a 1 ml. ethanolic solution was evaporated to dryness by heating in a stream of air. When the quinol required in the first stage of the colour reaction was added, the 'destruction' was only 8-10%. Since the urinary residues used in the experiment described in Fig. 4 were purified by alkalization of the initial ether extract, the indication was that the damaging agents were not completely removed by this step. Routinely, therefore, the ethanol used for the transfer of the final fractions to tubes for colorimetry was removed in the presence of the quinol required in the colour reaction.

4. *'Destruction' during extraction.* Recovery of oestrogen added to pure solution and taken through the extraction procedure was found to be nearly quantitative. Recovery from urine, however, was found to be incomplete especially for oestradiol, and a summary of the alterations in procedure necessary for improvement of yields is shown in Table 2.

Table 2. *Recovery of oestradiol added to acid-hydrolysed male urine*

Expt.	Oestradiol added to ½ aliquot hydrolysed male urine µg	No of determinations	*Procedure**	% Recovery *Mean ± S D.*
1	25	33	Alkalization of ether at stage (C)	61 ± 8.5
2	25	21	10 5 wash before alkalization of ether at stage (C)	71 ± 13.5
3	10	15	As in Expt 2	51 ± 8.2
4	10	10	As in Expt 2, but no washing in stage (H)	85 ± 5 8
5	2	12	As in Expt. 4	70 ± 6 1
6	1	8	As in Expt 4	81 ± 19 8
7	1	12	As in Expt 4, but with saponification of oestradiol eluate from chromatogram	84 ± 6 9

N B All recoveries are corrected for endogenous oestrogen content
*The basic method is outlined in Fig 1, and the changes made are noted below

Expt 1 demonstrated that a definite loss occurred on extraction of oestradiol-17β from acid-hydrolysed male urine when the procedure shown in Fig. 1 was followed. When the oestrogen (25 μg) was added to the urinary fraction immediately before chromatography, recoveries were improved (100, 96%; 88, 92% in two experiments). Evidently the major loss was occurring during *the preliminary purification* Since it is known that the oxidation of strongly reducing phenols may facilitate the oxidation of other substances [see Waters, 1946], it was thought possible that oxidative destruction of oestradiol-17β was occurring during the alkalization of the initial ether extract In model experiments it was shown that addition of quinol (1-2 mg) to the ether before the alkalization increased the loss of oestradiol-17β, whereas quinol added after the alkalization did not Moreover, the addition of quinol to a pure solution of oestradiol in ether, followed by alkalization, caused a 15-20% loss These experiments indicated the necessity for further purification of the ether extract before alkalization

Expt. 2 showed that when the ether extract was washed with a concentrated

Saponification, however, did effect a considerable purification of the oestriol fraction and, for convenience, was incorporated into the procedure at an earlier stage. The recoveries for oestrone and oestriol are shown in Table 3.

Investigations of the optimal conditions for hydrolysis are in progress. Most of the experiments were done with 1-amino-2-naphthol-4-sulphonic acid added to the urine before hydrolysis as suggested by Van Bruggen [1948], but no evidence of any protective action by this reagent has yet been obtained.

Typical analyses are shown in Table 4. In spite of the various purification stages, which remove more than 95% of the non-specific chromogens, the specificity of the method

Table 4. *Typical analyses of the oestrogen content of 24-hr specimens of urine*

Subject	Type	Oestrogen	E_1	E_2	E_3	E_{corr}	µg/24 hr
W.B	Male	Oestriol	0.249	0.222	0.134	0.030	
			0.296	0.262	0.166	0.031	8.2
		Oestrone	0.379	0.348	0.227	0.045	
			0.371	0.334	0.207	0.045	7.0
		Oestradiol	0.166	0.138	0.096	0.007	
			0.180	0.156	0.114	0.009	1.1
M F	Menopausal	Oestriol	0.112	0.097	0.079	0.006	
			0.032	0.027	0.018	0.002	1.0
		Oestrone	0.280	0.245	0.189	0.010	
			0.332	0.279	0.216	0.005	1.2
		Oestradiol	0.183	0.147	0.109	0.001	
			0.264	0.228	0.189	0.001	0.2
W.M	16th day of cycle	Oestriol	0.194	0.223	0.094	0.079	
			0.190	0.214	0.096	0.071	20.5
		Oestrone	0.416	0.409	0.249	0.076	
			0.439	0.418	0.244	0.076	11.4
		Oestradiol	0.268	0.268	0.200	0.034	
			0.258	0.257	0.187	0.034	6.0

E_1 = optical density at 480 mµ
E_2 = optical density at 512.5 mµ for oestriol and oestrone
at 515 mµ for oestradiol-17β
E_3 = optical density at 545 mµ for oestriol and oestrone
at 515 mµ for oestradiol-17β

at the low excretion levels shown in the table, is largely dependent upon the specificity of the Kober reaction. *The adequacy of the colour correction has already been discussed* in full by Dr Brown. Some of the background colour arises from the effect of solvent residues on the quinol added during the transfer of the final fractions to tubes for colorimetry, as is evident from blank analyses. Much of the residual chromogen, however, represents urinary residues which have not been removed in the purification procedures.

The method may readily be applied to the routine analyses of urine specimens. Four determinations are completed in 9–10 man-hr. A high resolving spectrophotometer is the only special piece of apparatus required. The procedures involved are simple enough to be carried out by a competent technician given 3–4 weeks of special training, provided he is under the close supervision of a research worker thoroughly familiar with the sources of error of the method.

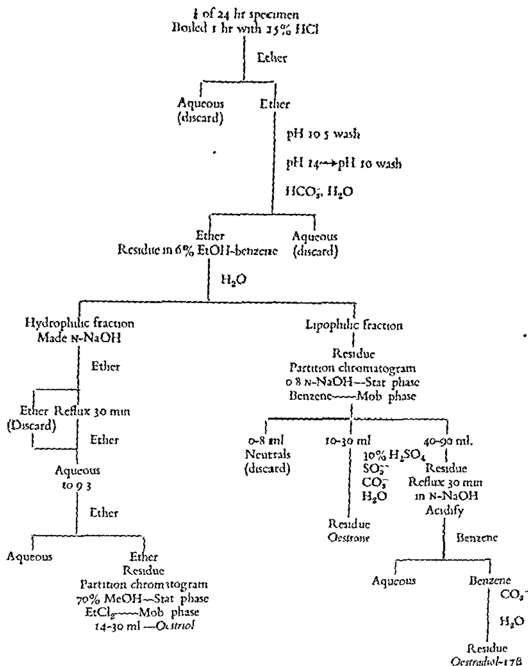


Fig 5 Outline of final method for the extraction and purification of oestriol, oestrone and oestradiol-17β from urine

THE SIGNIFICANCE OF COMPONENTS OF URINARY OESTROGEN FRACTIONS

By R. J. BOSCOTT
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SUMMARY

1 The question of the identity of natural oestrogens in human beings and the attempts to assess chemically the physiological oestrogen state of man are discussed

2 Paper chromatographic methods have been developed for the qualitative analysis of urinary oestrogens, the majority of the ether-soluble acidic and phenolic compounds and certain artefacts in hydrolysed urines

[illegible]

the composition of urinary oestrogen fractions.

The thesis that oestriol, oestradiol-17β and oestrone are the only compounds of interest in assessing the oestrogen state of human beings, both from the clinical and biochemical viewpoints, is still a matter of conjecture. Thus the compound isolated by [illegible]

some measure is required of the total oestrogen production by the ovaries, adrenal and placenta Lieberman & Teich [1953] in their review on the biochemistry of steroid hormones, suggest that attack of the benzenoid ring of the oestrogen molecule is an important step in the catabolism of oestrogens. This view is also supported by the preliminary report of Garst & Friedgood [1952] on the chemistry of the compound they have isolated.

Smith & Smith [1941; 1952] have published evidence that a urinary compound other than oestrone can be converted by treatment with zinc and hydrochloric acid into a product with increased oestrogenic activity. Furthermore, Smith & Smith [1952] have reported the presence of an unidentified labile oestrogen in the follicular fluid of women treated preoperatively with gonadotrophin. Previously, Huffman & Grollman [1947] suggested that 16-keto-oestradiol-17β may be the labile compound responsible for the [illegible]

such as 7-keto-oestrone, may be responsible for the higher oestrogenic activity after hydrogenation with the nascent hydrogen produced by the zinc. Similar theories could be postulated in relation to the unidentified oestrogen detected biologically in follicular fluid.

With regard to these suggestions, Marrian [1949] and Pincus [1949] have pointed out

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DISCUSSION

Dr Prunty. May I ask Dr Bauld if he has ever obtained a negative figure for the corrected extinction—in other words, is there any evidence that the absorption of his coloured background is not linear with some urines?

Dr Bauld Yes, we did obtain this earlier on in the method, particularly in the case of oestradiol That was one of the principal reasons why we had to introduce the final saponification stage *That is*, in the oestradiol determinations prior to the saponifications which we routinely do now, there was a negative correction of an extinction coefficient of 0 010–0.020 in certain urines.

Dr Prunty So you think there is reasonable evidence that linear extinction occurs over the range you are correcting on?

Dr Bauld With the residues as they are in the final method, yes.

Method C1. The extracts were enzymically hydrolysed (see method B), extracted and fractionated according to method A and Fig. 1.

Method C2. The extracts were hydrolysed with hydrochloric acid and worked up according to method A and Fig. 1.

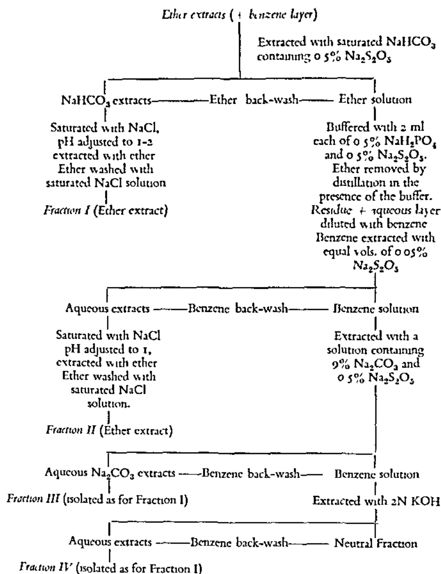


Fig. 1. Urinary fractionation flow sheet

Method D

The procedure of Engel [1950] was also used for the preparation of Fraction I and Fraction V (Engel's oestrogen fraction). Saturation of the hydrolysed urine with NaCl

that the influence of possible synergists or antagonists of oestrogenic activity may invalidate conclusions derived from experiments based upon bioassay figures. In addition, Marrian & Bauld [1951] have criticized the claims of Smith & Smith [1941].

Values for the ratio of urinary oestrogens to oestrogen antagonists or their metabolites (rather than oestrogen values alone) may give a truer biochemical assessment of the general, physiological oestrogen state in man. With this principle in mind, Salter, Humm & Oesterling [1948] measured ratios of urinary oestrogens ('oestroids') to neutral 17-ketosteroids in man. Szego & Roberts [1953] have shown that cortisone and cortisol (Kendall's compound F) exhibit antagonism towards the biochemical and physiological effects of oestrogen on the uteri of ovariectomized rats. Since improved methods for the quantitative estimation of oestrogens and other steroidal metabolites are now available [Engel, 1950; see also review by Lieberman & Teich, 1953], the work of Salter *et al.* [1948] should be extended to include ratios of the urinary adrenocortical steroids to oestrogens.

The objects of the present work were:

(i) Development of improved methods for the extraction of urinary oestrogens, acidic and phenolic compounds

(ii) Complete analyses by paper chromatography of urinary extracts containing these compounds.

(iii) Study of factors influencing the composition of the urinary extracts.

(iv) Investigation of the effects of acid hydrolysis conditions on acid-labile oestrogen-oestradiol-17α.

METHODS

I. *Preparation of extracts*

Twenty four-hour collections of urine were preserved by the addition of 5 ml. of both chloroform and acetic acid. Six-hr aliquots of urine were hydrolysed according to Methods A, B, C_1, C_2, D or E (below).

Method A

Six-hour aliquots of 24 hr collections of urine were subjected to hydrolysis by boiling under a reflux condenser for 30 min after the addition of 15 vols. % concentrated HCl, and a small volume of benzene. The cooled hydrolysed urine was saturated with NaCl and extracted four times with its quarter volume of ether. The ether extracts were fractionated, yielding Fractions I-IV as shown diagrammatically in Fig. 1.

Method B

Urines were boiled for 10 min at pH 5, buffered to pH 6 2, and incubated for 36 hr with bacterial glycuronidase—(Sigma Chemical Company), using 100 mg/400 ml. of urine. Enzymic activity after the 36 hr hydrolysis period was checked by incubation of a sample with phenolphthalein glycuronide. The hydrolysed urine was then adjusted to pH 1 and fractionated according to Method A and Fig 1.

Methods C1 and C2

Aqueous solutions of urinary steroidal conjugates were prepared according to the method of Edwards, Kellie & Wade [1953]

RESULTS

(A). *Chromatography of Fraction I (Method A)*

An aliquot of urine equivalent to from $\frac{1}{200}$ - $\frac{1}{2000}$ of a 24 hr collection of human urine was used.

Fig. 2 and the accompanying key represents the total number of compounds found in 200 human urines, which were detectable with tests 3-5. Far fewer compounds were detected on chromatograms of Fraction I obtained from human patients and rats fed highly purified diets (essentially free from vegetable components) than when a more complex diet was given. It is interesting to note that a methylated phenolic acid—vanillic acid—was detected in Fraction I (method A—purified diet), thus recalling the work of Maclagan & Wilkinson [1951] on the *in vivo* methylation of n-butyl 3 : 5-diiodo-4-hydroxybenzoate.

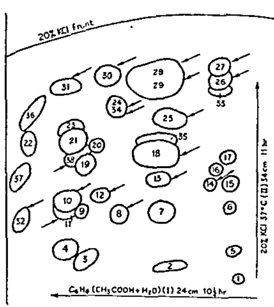


Fig. 2 Map of phenolic acids found in Fraction I

Key

Colour of the dyes (Test 4)
Compounds 5, 6, 17—yellow
Compounds 15, 16, 25—orange-yellow
Compounds, 2, 7, 18, 19, 22, 24, 27, 28, 33, 37—orange
Compound 8—orange-pink
Compounds 12, 14, 20—pale pink
Compounds 3, 10, 21, 26, 29, 32, 34—red
Compound 9—magenta
Compounds 11, 23, 31—violet
Compounds 4, 13—mauve

Compound 36 (Test 5)—yellow fading to greyish blue
Compound 38 (Test 2)—green fluorescence becoming pink-magenta in colour on long standing or after Test 3

was used to depress the formation of emulsions during the extraction of the urine with ether.

Method E

Method C1 (enzymic hydrolysis) but with fractionation according to method D is under investigation

II. *Paper chromatography*

A. *Techniques employed*

Some of the paper chromatographic methods used have been described briefly elsewhere [Boscott 1952, 1954; Boscott & Bickel, 1953; Boscott & Cooke, 1954]. The essential features of the methods used are recorded in Figs. 2-8. Whenever possible, solutions of electrolytes were used instead of organic solvents for the development of the paper chromatograms. The use of electrolyte solutions saturated with an organic solvent —see Figs. 2 and 7— as 'single phase' systems prevents the formation of diffuse oestrogen zones obtained when only the electrolyte solution is used. A solution of 5% sodium formate in water at room temperature may be used instead of 20% KCl at 34°C for the chromatography of Fraction I.

Paper impregnated with a salt or other compound which is appreciably soluble in one component of a non-aqueous developing solvent mixture may be used for the chromatography of many compounds with poor solubility in aqueous stationary organic solvents commonly used as the stationary phases in conventional paper chromatography.

The hydrotropic agent, sodium p-toluenesulphonate, is appreciably soluble in absolute methanol, but only slightly in toluene. Whatman papers Nos. 542 or 3MM were therefore sprayed with a half-saturated aqueous solution of sodium p-toluenesulphonate, leaving a 1 in margin unsprayed. The papers were dried and the urinary oestrogen extracts applied to the paper in acetone solution as narrow bands 1 in. from the unsprayed margin of the paper The unsprayed edge of the paper was placed in the developing solvent—1 vol absolute methanol to 9 vols. toluene—without previous equilibration; chromatographic development then proceeded by the capillary ascent technique.

Using paper No. 542, chromatogram development took 12 hr for 30 cm development, whereas the thicker paper No. 3MM took only 5 hr. An extension of this technique shows promise for the chromatography of neutral steroids and may be regarded as a development from the methods of Sakal & Merill [1953] and Boscott [1952]

B. *Tests for locating compounds on chromatograms*

Each chromatogram was investigated by the following tests in the order given.

1 Fluorescence of the compound at neutral pH in UV light (Mercury vapour lamp with Wood's glass filter)
2. Fluorescence and colour development with microdrops (1 mm diameter) of 90% (v/v) H_2SO_4 spaced at suitable intervals.
3. Fluorescence and colour changes after spraying with 9% Na_2CO_3 solution
4. Formation of azo dyes by spraying with a sodium carbonate solution of diazotised p-aminophenyldiethylaminoethylsulphone (I.C I. 5091).
5. Colour changes after spraying with 2N-KOH shortly after test 4.

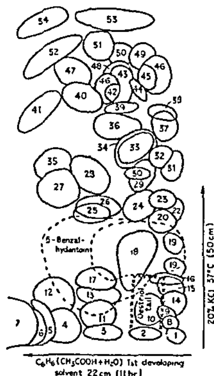


Fig 3 Map of compounds found in Fraction II, Method A, human urine Capillary ascent technique

Key to Fig 3*

Spot Number	Test Number 1	2	3	4	5
	Reaction given				
1	BR(C) PV(C)	YG(F) Y(C)	OR Y(F)	—	—
2 Oestriol	G(F)	{G(F) P(C)		OR	PV
3	V(C)	—	—	—	—
4	B(F)	—	—	—	—
5.				R	—
6	OR(F)	—	—	OR	—
7	Y(F)	—	—	—	—
8	B(F)	—	—	—	—
9	B(F)	—	—	—	—
10	B(F)	—	{BG(F) Y(C)	—	—
11	G(F)	—	BW(F)	—	—
12	BV(F)	—	—	—	—
13	OR(F)	—	R(C)	—	—

Key of Fig 2 (continued)

Identity of the compounds, where known

Compound	
4	—ferulic acid
10	—vanillic acid
14	—5-hydroxy-β indolylacetic acid (tentative)
18	—4-hydroxybenzoic acid
24	—[illegible]
26	[illegible]
28	[illegible]
29	[illegible]
30	[illegible]
34	—4-hydroxyphenylpropionic acid
36	—phenylpyruvic acid
38	—β-indolylacetic acid

The significance of the tyrosine and phenylalanine metabolites Nos. 24, 26, 28, 29 and 34 has been reported previously or is in press [Aterman, Boscott & Cooke, 1953; Boscott & Bickel, 1953; Boscott & Cooke, 1954]. The excretion of these compounds is influenced by conditions with abnormal ascorbic acid metabolism, arthritis, gastro-intestinal disturbances, certain anaemias, pregnancy and liver disease. The excretion of compound 25 was found to be greatly increased in a case of essential hypertension. Compound 36 was observed only in phenylketonuric urine, as also was the vastly increased urinary excretion of compound 30. The tryptophane or tyrosine metabolite [Robinson, 1952] 5-hydroxy-β-indolylacetic acid (compound 14) has been observed in greatly increased amounts in urines from a small number of patients with both steatorrhoea and liver disease as well as one cancer patient with metastases in the liver. Compound 38—β-indolylacetic acid—was detected (test 2) in abnormally high amounts in chromatograms of urines from patients with steatorrhoea and other gastrointestinal disorders

Marrianolic acid has not been detected in Fraction I of the late pregnancy urines examined. If present in amounts of from 0.5-1 mg/24 hr specimen, it would have been detected close to compound 8, and given test reactions similar to oestriol (Figs. 3 and 4)

(B). *Chromatography of Fraction II (Methods A, B, C1 and C2)*

An aliquot of from one-tenth to one-fortieth of a 24-hr urine collection was used for the paper chromatography by the methods indicated in Figs. 3 and 4. The type of compounds detected is indicated in the keys accompanying Figs 3 and 4. Fraction II (method A) contained many more components than Fraction II (methods B, C1 and C2)

Fraction II (method B and C1), chromatographed on paper strips using 10% KCl saturated with N-hexyl ether as developing solvent, contained several compounds giving positive reactions with alkaline tetrazolium reagents. With the chromatographic developing solvent used cortisone and cortisol are known to have Rf values considerably higher than oestriol. Oestriol was detected readily in late pregnancy urines by this method, concomitant purple and red pigments being easily separated chromatographically.

Five-substituted hydantoins arising as artefacts from the condensation of urea with certain a-ketoacids may occur in Fraction II (method A) but not in Fraction II (methods B and C) [Boscott & Bickel, 1953] The chromatographic detection of some of these compounds is shown in Fig. 4 5-(4-hydroxybenzal)-hydantoin arising from urea and

4-hydroxyphenylpyruvic acid was found in abnormal amounts in chromatograms of acid hydrolysed urines from most patients with disturbed ascorbic acid metabolism, nutritional deficiencies, toxaemia of pregnancy and liver disease. Indeed, Felix [1944] has employed test doses of 4-hydroxyphenylpyruvic acid as a liver function test in humans.

A number of compounds give green fluorescence and pink colours with 90% sulphuric acid (test 2) reminiscent of the Kober reaction for oestrogen (see Fig. 3). The effect of dilute HCl on the compounds detected would serve to differentiate oestrogens and other compounds from those giving a green fluorescence merely in response to a change in pH. The interaction of the compounds detected, with the cellulose of the paper and the acid used, may in part be responsible for the reactions given.

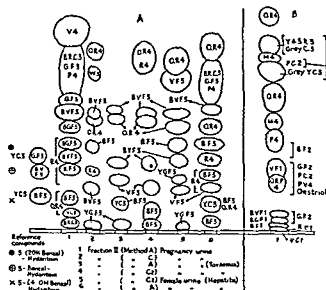


Fig 4 Chromatography of urinary oestrogens in Fraction II
A. Whatman paper No 542 Developing solvent—2% K_2CO_3 (31 cm) Capillary ascent technique
B Developing solvent—10% KCl in water saturated with N-hexyl ether
Key 2, 3, 4, 5, Test numbers, F—Fluorescence SH—Shadow, C—Colour, O—Odour, B—Blue, BG—Bluish green, BV—Bluish violet, BR—Brown, G—Green, P—Pink, R—Red, OR—Orange, OR-P—Orange pink, V—Violet, Y—Yellow, M—Mauve, SR—Salmon red, SW—Sweet, WB—Whitish blue

The polyphenolic compounds 29 and 43 (pyrocatechol, method A) are excreted generally in abnormal amounts by patients with nutritional deficiencies, liver disease, aplastic, microcytic and refractory megaloblastic anaemias. Compounds 41, 52, 53 and 54 are frequently obtained from the urines of patients with steatorrhoea (method A).

Interest attaches to the presence of resorcinol, compound 46 (yellow dye test 3) in Fraction II, in view of its antithyroid properties [Arnott & Doniach, 1952] Relatively large amounts of resorcinol are found occasionally in urines.

Rf values of polyphenolic compounds are given in Table 1.

Spot Number	Test Number 1	2	3	4	5
14	BV(F)	PV(C)	{B(F) Y(C)}	5-p-hydroxybenzalhydantoin (spots 14–15, test 3) —	—
15.	BV(F)	—	{B(F) Y(C)}	—	—
16	B(F)	—	YG(F)	—	—
17	BV(F)	—	—	—	—
18.	BV(F)	YG(F)	BF	—	—
19.	—	{P(C) G(F)}	—	—	—
20	—	G(F)	—	—	—
21	—	G(F)	—	R	—
22.	BN(F)	YG(F)	—	—	—
23	—	—	—	GR	M
24	V(F)	B(F)	BV(F)	OR.P	—
25	—	—	V(F)	—	—
26	—	G(F)	—	—	—
27	V(F)	—	V(F)	—	—
28	—	—	BG(F)	R	—
29	—	—	—	GRM	M
30	V(F)	—	—	—	—
31	B(F)	—	B(F)	—	—
32	—	—	BR(C)	—	—
33	—	—	BV(F)	—	—
34	—	—	R(C)	—	—
35.	—	—	V(F)	OR	—
36	—	—	GR-BR(C)	—	—
37	—	—	R(C) R(F)	—	—
38	—	—	BV(F)	—	—
39	—	—	S(C)	V	—
40	V(F)	G(F)	B(F)	—	—
41	G(F)	—	—	—	—
42	—	R(C)	—	—	—
43 Pyrocatechol	—	—	BG(C)	—	—
44	—	—	BG(F)	—	—
45	—	—	{B(F) Y(C)}	Y	S
46	—	YG(F)	G(F)	Y	S
47	—	V(F)	—	—	—
48	—	—	V(C)	—	—
49	—	—	Y-BR(C)	—	—
50	—	—	R(C)	—	—
51	—	—	—	OR	—
52	G(F)	—	—	—	—
53	G(F)	—	—	—	—
54	G(F)	—		—	—

*For abbreviations see Fig 4

Key of Fig 5.*

Spot Number	Test number 1	2	3	4	5
	Reaction given				
1	BR(C)	—	—	—	—
2.	—	G(F)	—	—	—
3.	G(F)	G(F)	—	—	—
4.	—	—	—	S OR	—
5	BG(F)	—	YG(F)	—	—
6	BG(F)	G(F)	—	—	—
7.	—	—	—	R	—
8.	B(F)	—	—	—	—
9	OR(F)	G(F)	—	—	—
	Y(C)				
	U(O)				
10	—	—	BV(F)	—	—
11.	—	—	Y(C)	—	—
12	—	—	B(F)	—	—
13.	BV(F)	—	—	—	—
14	—	—	—	R	—
15.	OR(F)	—	R(C)	—	—
16.	BV(F)	—	B(F)	—	—
			BG(C)		
17.	OR(F)	—	P(C)	—	—
18.	B(F)	—	—	—	—
19	—	—	B(F)	—	—
20	—	—	B(F)	—	—
21	B(F)	—	—	—	—
22	—	—	—	OR	—
23.	—	—	—	R	—
24	—	—	V(F)	—	—
25	V(F)	—	—	—	—
26	—	—	G(F)	—	—
27	G(F)	—	—	—	—
28	SH	—	—	—	—
29	—	—	B(F)	—	—
30	—	—	V(F)	—	—
31	G(F)	—	—	—	—
32	SW(O)	—	—	—	—
33	G(F)	—	—	—	—
34	—	—	—	OR	—

*For abbreviations see Fig 4

compound 4 in relation to liver disease In general, chromatography of Fraction III (method A)—from liver disease patients—revealed the presence of a larger number of fluorescent compounds than was found in Fraction III (method A) normal urines. Occasionally three compounds giving on paper a green fluorescence with 90% sulphuric acid, similar to oestrogens, were detected in this fraction. The possibility that the partition coefficient of oestradiol and oestrone distributed between benzene and 9% sodium

Table 1. *Rf values of polyphenolic compounds*

Paper: Whatman No. 542
Developing solvent: 10% KCl solution in water
Distance moved from origin, by solvent: 50 cm

Compound	Rf value	Method of Detection
Hydroquinone	0.73	Test 3. Brown colour
Resorcinol	0.70	Test 4. Yellow; Test 5. Salmon
Pyrocatechol	0.69	Test 3. Blue-green colour; Test 4. Violet halo
4-Methylcatechol	0.66	
Toluhydroquinone	0.64	Test 3. Brownish-violet colour
3-Methylcatechol	0.64	Test 3. Brown colour; Green fluorescence
Pyrogallol	0.62	Test 3. Brown colour
Orcinol	0.60	Test 4 Yellow; Test 5. Yellow
Phloroglucinol	0.53	Test 3 Blue-violet fluorescence; Test 4. Yellow
Protocatechuic acid	0.49	Test 3. Violet fluorescence; Test 4. Magenta
Gallic acid	0.38	Test 3. Yellow colour green fluorescence; Test 4 Brown
Naphthoresorcinol	0.22	Test 3. Orange colour, blue-violet; Test 4. Yellow

(C). *Chromatography of Fraction III (methods A, B, C1 and C2)*

The compounds found in Fraction III (method A) are illustrated in Fig. 5. Fraction III (methods B, C1 and C2) contained fewer components than Fraction III (method A). Comments have been made [Aterman, Boscott & Cooke, 1953] on the significance of

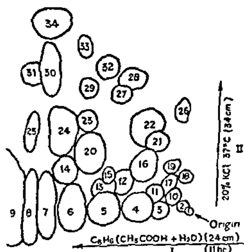


Fig 5 Map of compounds in Fraction III, Method A, Whatman paper No 542

carbonate may be modified by the hydrotropic action of abnormal amounts of other compounds extracted by sodium carbonate, should be considered, especially in view of the comments by Bates [1952] on co-solubility phenomena influencing the extraction of oestrogens from organic solvents.

(D). *Chromatography of Fraction IV (methods A, B, C1 and C2) and Fraction V (method D)*

Representative chromatograms of these fractions are illustrated in Figs. 6, 7 and 8.

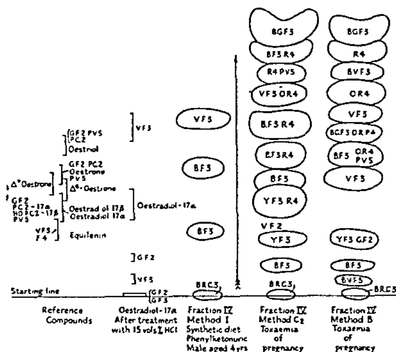


Fig 7 Chromatograms of urinary oestrogen fractions and reference compounds Whatman paper No 542 Developing solvent—5% K_3PO_4 in water, saturated with N-butanol Capillary ascent technique (30 cm) (For key, see Fig 4.)

Rf values of the oestrogens with two of the solvents used are given in Table 2.

Equilenin is valuable as a reference marker on the chromatograms in view of the strong violet fluorescence it gives with alkali (test 3) and the characteristic red colour (test 4)

Some nineteen components have been observed in Fractions IV (method A) and V

[illegible]

these fractions.

The chromatographic methods illustrated in Fig. 6 were used primarily for the detection of compounds accompanying the oestrogens rather than the oestrogens themselves, which were not separated in sufficiently restricted zones Compound 17 (Fig. 6)

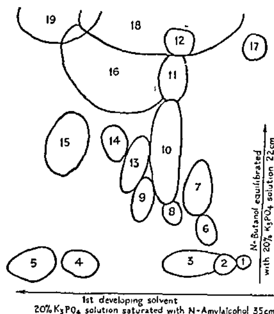


Fig 6 Map of compounds in Fraction V, Method D, obtained from human pregnancy urine

Key of Fig 6 *

Spot Number	Test Number 1	2	3	4	5
1	BR(C) PV(C) OR(F)	G(F)	—	—	—
2	OR(F)	—	—	R	—
3	G(F)	—	—	S OR	—
4	B(F) BRC	— —	— —	— —	— —
5	BV(F)	—	—	—	—
6	BV(F)	—	—	—	—
7	BV(F)	—	—	—	—
8	B(F)	—	—	—	—
9	B(F)	—	—	R	—
10 Oestriol	— —	G(F) R(C)	— —	OR —	PV —
11 Oestradiol-17β	—	G(F)	—	OR	PV
12 Oestrone	— —	G(F) R(C)	— —	OR —	PV —
13	V(F)	YG (weak)	—	—	—
14	BG(F)	—	—	—	—
15	—	—	—	—	PV (faint)
16	—	—	—	OR	—
17	BR-Y (faint)	Y(C) P(C)	W-BF	—	PV
18	—	—	—	P	PV
19	SW(O)	—	—	—	—

*For abbreviations see Fig 4

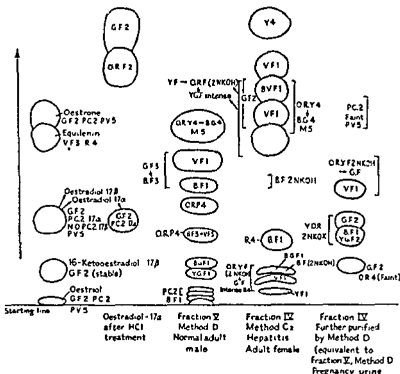


Fig 8 Chromatograms of urinary oestrogen fractions and reference compounds Whatman paper Ny 542, impregnated with Na-p-toluene sulphonate Developing solvent—9 vols toluene + 1 vol. methanol Capillary ascent technique (30 cm), 5 hr
*For abbreviations see Fig 4

fluoresced a brilliant sky blue are illustrated in Figs. 7 and 8. Further experiments of a similar nature may lead both to a better understanding of colour and fluorescence reactions for the steroidal oestrogens and to the recognition of factors leading to the destruction of oestrogens during urinary acid hydrolysis or extraction procedures.

DISCUSSION

Judging by the variations in composition of human urines, it would appear improbable that consistently good recoveries would be achieved of 10-200 μg amounts of steroidal oestrogens added to the urine prior to acid hydrolysis The different amounts of protein, carbohydrate, polyphenolic compounds, iron and copper encountered in urines of patients with different pathological conditions might influence the degree of destruction of urinary oestrogens subjected to hydrolytic and extraction procedures. Indeed, the addition of zinc to the acid hydrolysis medium [Smith & Smith, 1941] would destroy highly reactive carbonyl compounds—for example, quinones, and aldehyde derivatives from sugars—which would otherwise react with the phenolic oestrogens The zinc would also remove traces of copper present which would tend to catalyse the oxidation of polyphenolic and other compounds to reactive quinones

Table 2. *Rf values of oestrogens*

Compound	Rf values — Method: Whatman Paper No 542. *Developing solvent*—5% K_3PO_4 saturated with N-butyl alcohol	Rf values — Method: Whatman paper No 3MM impregnated with sodium p-toluene sulphonate. *Developing solvent*—Toluene-methanol (9 : 1)
Oestriol	0.40	0 02
16-Keto-oestradiol-17β	0.40	0 11
Oestradiol-17α	0 30	0 21
Oestradiol-17β	0 28	0 21
Oestrone	0 35	0 50
Δ^6-Oestrone	0 26	0 47
Equilin	0 30	0 47
Equilenin	0 22	0.46
Violet pigment (Fraction IV (Method A))	0 00	0.08

may conceivably give a colour with the Kober reagent in view of its reaction with 90% *sulphuric acid on paper (test 2)*

Fig 7 shows diagrammatically chromatograms of urinary oestrogen fractions, the components being detected by tests 2, 3 and 4. The developing solvent was prepared by equilibrating 300 ml. of 5% K_3PO_4 solution with 100 ml. of n-butanol. The resulting more aqueous layer was used as the mobile phase.

Fig. 7 illustrates the use of paper impregnated with sodium p-toluenesulphonate and a non-aqueous organic solvent mixture for the chromatography of Fractions IV and V. Tests 1-5 were applied to the chromatograms A compound closely resembling 16-keto-oestradiol-17β in Rf value, and fluorescence reactions (test 2) was detected in Fraction IV (method C2) late pregnancy urine, but not in urines from a small series of normal individuals and from one patient with hepatitis. Other compounds were also detected in the same zone as the 16-keto-oestradiol-17β like material by tests 1 and 3

(E) [illegible]

[illegible] oestrogen with acid for 30 [illegible] udied chromatographically (Figs. 7 and 8). The acid solution of the oestrogen rapidly assumed a pink colour during boiling and fluoresced a brilliant green in UV light Towards the end of the heat- [illegible] on cooling The colour reaction so obtained [illegible] was saturated with NaCl and extracted [illegible] ts which

Samples of the many reference compounds used in these studies were donated by Dr K. Miescher (Ciba), Dr C. Djerassi (formerly Syntex), Dr W. J. Tindall (Organon), Dr F. Hartley (B.D.H. Ltd.), Dr E. B. Hershberg (Scherings, U.S.A.), Dr M. Huffman, Prof. E. Lederer, Dr J. B. Jepson, and Dr H. G. Bray.

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DISCUSSION

Dr Hannelore Braunsberg: Have you found that samples of commercial oestriol contain any fluorogenic impurity?

Dr Boscott. In our samples, as far as I remember, we only get one component, but we have seen trace impurities in some of the other oestrogens. We find, for example, some traces of equilenin in some of the oestrone samples, and we found a trace impurity in a sample of Δ^6-oestrone, and also a trace impurity in our 16-keto-α-oestradiol. I also recall having separated from crude oestriol obtained from pregnancy urine, a few crystals of a compound with a melting point at least 2°C higher than that of oestriol. This compound gave colour and fluorescence reactions very similar to oestriol. More recently we paper-chromatographed this material and found that this 'oestriol' always produced a tail, whereas pure oestriol formed a nice compact spot. We suspect that the tail may be ascribed to part of the impurity present.

Dr Swyer: That is most interesting to us. We never succeeded in obtaining a sample of

Veldhuis [1953] has reported good recoveries of oestriol from a few micrograms of oestriol glycuronide added to plasma and hydrolysed with bacterial glycuronidase The present writer has encountered some urines which inactivate β-glycuronidase more than others It is therefore advisable to test the urine (with phenolphthalein glycuronide) for remaining enzymic activity after the standard period of enzymic hydrolysis has taken place. The addition to the urine of the enzyme in several portions at intervals, rather than all at once, appears to be advantageous. The use of an inert atmosphere in the enzyme hydrolysis vessel is recommended to obviate the autoxidation of the liberated urinary components. The enzymic method of hydrolysis of oestrogen conjugates used in conjunction with the method of Edwards, Kellie & Wade [1953] for the extraction of urinary steroidal conjugates, may prove to be the method of choice in the study of urinary oestrogens, since the destruction of labile compounds would be minimised, and few artefacts would be formed. A disadvantage of the enzymic procedure appears to be an increased contamination of the oestriol (Fraction II) with compounds—presumably adrenocortical steroidal metabolites—giving positive reactions with alkaline tetrazolium reagents. The use of Engel's oestrogen extraction procedure [1950] would minimize contamination of this nature, but would lead to destruction of alkali-labile oestrogens if such exist. Method II, C2 is preferable to method A if a non-enzymic hydrolysis technique is resorted to Fewer artefacts are then produced.

The smaller number of components found in Fractions I-IV from human patients and experimental animals fed synthetic or purified diets leads to the conclusion that these diets should be given when metabolic studies on isotopically-labelled oestrogens are undertaken. The identification and recognition of the urinary non-steroidal metabolites of oestrogens would then prove less difficult, since minimal numbers of extraneous compounds unrelated to normal body constituents would be encountered.

In the present studies, several urinary components have been identified by classical means, others have been recognized by feeding specific precursors. Paper chromatography using tests 1-5 and supplementary microchemical tests, and the use of reference compounds have led to the tentative identification of the compounds detected in human urine.

The studies on oestradiol-17α may prove of interest in relation to the mechanism of the Kober and fluorescence reactions for oestrogens. Further, similar studies are in progress on the compounds produced when oestrogens are treated with mineral acids.

An extension of the work of Slaunwhite, Ekman, Engel, Nathanson, Pincus & Carlo [1951], but based on paper chromatographic rather than countercurrent separation of oestrogen fractions, and their bioassay, are required for a more complete understanding of the nature of urinary oestrogens.

The author wishes to thank Prof S Zuckerman for encouragement in this research

The clinical material was kindly made available by physicians of the United Birmingham Hospitals Group, in particular Prof. H. C. McLaren, Prof A. P. Thomson, Dr W. T. Cooke, Dr H. Bickel, and by Dr K. Aterman. Dr C. E Dent (University College Hospital, London), provided urines from a child receiving a synthetic diet.

EXPERIENCES WITH OESTROGEN DETERMINATION BY FLUORIMETRY AFTER SEPARATION BY PARTITION CHROMATOGRAPHY

By HANNELORE BRAUNSBERG, MARGARET I. STERN *and* G. I. M. SWYER

From University College Hospital Medical School, London, W.C.1

METHOD

The method used in our laboratory for the determination of oestrogens in body fluids consists of three parts:

I. Hydrolysis, extraction and partial purification by partition between several pairs of solvents

II Further purification of the phenolic material by partition chromatography on a celite column.

III. Fluorimetric estimation of oestrone, oestradiol and oestriol in the three fractions obtained.

The technique will first be outlined, and then the significance of the results we have so far obtained will be discussed

TECHNIQUE

For urine, the hydrolysis and extraction used is essentially that described by Finkelstein [1953]. The oestrogens are liberated from their conjugates by boiling with concentrated hydrochloric acid (15 vols / 100 vols urine) and are extracted with ether, after saturating the aqueous phase with sodium chloride. The ether extract is washed to remove strongly acidic compounds, reduced to a small volume, and extracted with aqueous N/1 caustic soda. The pooled aqueous extract is adjusted to pH 8-9 and extracted with ether. The residue obtained after evaporation of the ether contains the weakly acidic material including phenols.

The phenolic residue is further purified by partition chromatography on a celite column containing aqueous caustic soda as stationary phase The solvents used for elution have been modified to yield purer oestrogen fractions. Instead of benzene [Swyer & Braunsberg, 1951] a mixture of benzene and petroleum ether (4 1) is at present being used for the elution of oestrone and oestradiol, and the 3 1 chloroform-butanol mixture [Stern & Swyer, 1952] has been replaced by a 17 3 chloroform-butanol mixture. The elution pattern was found to vary with temperature, and chromatogaphic separation is now carried out at 22° ± 0 5°C The flow rate should not be faster than approximately 0.5 ml./min to ensure equilibrium on the column. Fractions of approximately 4 ml. are collected by a mechanical automatic fraction collector.

A preliminary fluorimetric analysis ('fractional marker assay') is carried out on a small aliquot, usually 1/8 of each fraction. By this means we are able to ascertain which of the

pure oestriol. We suppose that all oestriol samples produced from pregnancy urine are impure, no matter how pure they are alleged to be. We wondered whether we could at some stage of our own work obtain some synthetic oestriol, which might be free from this troublesome contaminant, as we suppose it to be.

Miss Stern: Might I add that we would be very glad to have some of this oestriol of Dr Boscott's, so that we could chromatograph it and see if we get the same effect with our own samples, and find out if it is just one substance

Dr Hannelore Braunsberg: I would go a step further and ask for some of your crystals of contaminant, too, for comparison.

Dr Boscott: They are not absolutely free from other compounds; we have detected about five or six trace impurities in the unidentified oestriol contaminant.

the highest and lowest quantities found during the proliferative and the progestational phases respectively are given (Table 3). In the same table are shown the results of oestrogen determinations on the urines of various other female subjects.

Table 3. *Urinary excretion of oestrogens by female subjects* (µg/24 hr)

Subject	Oestrone	Oestradiol	Oestriol
Simmond's disease	3.3*	None detected by fractional marker assay	None detected by fractional marker assay
Oligomenorrhoea	3.9*	Very small amount detected	None detected by fractional marker assay
Jaundice, aged 44 years; spider naevi, hydatid cyst (proliferative phase of cycle)	*Free* 2.5 *Total* 16.4	0.3 1.3	9* 11.3*
Amenorrhoea, aged 25 years, tall, obese, acromegalic features	14	2.9	34
Normal female			
proliferative phase (range)	3.9–11.5	Nil–1.7	5–41
progestational phase (range)	3–19.1	0.5–4.3	5–97
Adipose gynism	2.9	1.0*	None detected by fractional marker assay
Obesity, virilism, aged 35 years	15	2.5*	8*
Post-menopausal	4.3*	None detected by fractional marker assay	19.5*

*Evidence of quenching and/or non-specific fluorescence

In pregnancy, the urinary oestrogens are present in greatly increased amounts, so that methods far less sensitive than ours may be quite adequate. Indeed, before accurate fluorimetry can be undertaken, more than a thousand-fold dilution of the chromatographed extracts may be necessary, even when only 50 ml of urine have been processed. Table 4 shows some of the results we have obtained

An attempt has been made to estimate the oestrogen content of a sample of ovarian cyst fluid. After acid and alkaline hydrolysis, followed by extraction and chromatography, no oestrogen could be detected fluorimetrically. To another aliquot 20, 30 and 50 µg of oestrone, oestradiol and oestriol respectively were added, followed by the same processing, recoveries of 20–50% only were obtained. The reasons for these losses are being investigated for they are most germane to the problem of blood oestrogen assay.

strated; four showed some quenching but no 'non-specific' fluorescence; while twenty-four showed quenching and the presence of some fluorescent impurity Of twenty determinations on pregnancy urines, thirteen were satisfactory, two were quenched without non-specific fluorescence, and in five there was both quenching and non-specific fluorescence. Finally, of eleven determinations on male urines (including those of two boys) only two were free from interference and nine showed quenching in the presence of fluorescent impurities. We are still inclined to be rather suspicious of the results of any determinations in which interference with fluorescence can be demonstrated, though the errors may sometimes be quite small. Further attempts to obtain purer fractions—such as the use of improved solvent mixtures for chromatography already mentioned—may lead to a high proportion of satisfactory results. With a highly sensitive fluorimeter it is sometimes possible to 'dilute out' any quenching substances.

RESULTS

Table 2 gives the results of oestrogen determination on the urines of various male subjects. Clearly, many 'normal males' are needed before an adequate baseline is established Moreover, determinations on successive daily outputs must be made before the day-to-day variation can be assessed

Table 2 *Urinary excretion of oestrogens by male subjects* (μg/24 hr)

Subject and age	*Oestrone*	*Oestradiol*	*Oestriol*
African (28 years)**	5.5*	None detected by fractional marker assay	5 1*
African (29 years)	1 1*	,, ,,	15 6*
African (39 years)	7 8*	,, ,,	33
Boy (9 years), Adipose gynism	2 7	1 8*	None detected by fractional marker assay
Boy (12 years), Adipose gynism	approx 4	1 3*	,, ,,

*Evidence of quenching and/or non-specific fluorescence
**Urine extracted with carbon tetrachloride, a poor solvent for oestradiol and oestriol

So far we have been able to carry out only a pilot survey of the urinary oestrogen excretion during the menstrual cycle. Since our methods have been undergoing con-

Finkelstein, M., Hestrin, S & Koch, W (1947) *Proc Soc exp Biol, N Y*, 64, 64
Stern, M I & Swyer, G I M (1952) *Nature, Lond*, 169, 796
Swyer, G I M & Braunsberg, H (1951) *J Endocrin* 7, lx

DISCUSSION

Miss Harlow: May I ask Dr Swyer how he detected the background fluorescence between oestrone and oestradiol. How do you know that it was not one of those two compounds? Is it a different colour?

Dr Swyer: With pure oestrone and oestradiol there was practically no background fluorescence between them at all.

Miss Stern: I think in this case there was only one eluate tube between the separations, and it probably contained a mixture of a small amount of oestrone and a small amount of oestradiol overlapping.

Miss Harlow: We also found the different substance Dr Braunsberg mentioned with the oestriol (from Organon). How do you account for the higher than 100% recovery of oestriol?

Dr Swyer: I am not sure that we can I would regard it as an experimental error.

Miss Harlow: We have found that also. It is rather surprising, seeing that part of the oestriol added was removed with the greenish background fluorescence.

Dr Swyer: It all depends upon this question of what appears to be non-specific fluorescent material which contaminates the oestriol, and until we are able to chromatograph some really pure oestriol, I do not think we shall have the answer to that part of the problem.

Table 4. *Urinary excretion of oestrogens in pregnancy*

Subject and duration of pregnancy (weeks)		Oestrone (μg/24 hr)	Oestradiol (μg/24 hr)	Oestriol (mg/24 hr)
	22	180	11	3.3*
Krohn's disease	22	40*	0.3	2.5
	27	108*	32.5*	5.65*
	30	204	19	12.35
	33	240	124*	12 (approx.)
Pre-eclamptic toxaemia	28	193	35.5*	1.25
	34	230*	36	14.7*

*Evidence of quenching and/or non-specific fluorescence

DISCUSSION

Further studies must be directed, not only towards obtaining far more values in normal and abnormal patients, but also to a number of technical details. We lack data on the optimum conditions for hydrolysis and extraction. Studies involving the addition of known amounts of conjugated oestrogens, before hydrolysis and extraction, are still needed. Although our present first mobile phase in the chromatography procedure effects a better separation of oestrone and oestradiol, the former still tends to be eluted too early so that the oestrone fraction from some extracts may be contaminated. Further simplification of the extraction and chromatographic procedure would obviously be very desirable, though it is difficult to see how it can be achieved without loss of accuracy.

The use of a monochromator and a more intense source of light in the actual measurement of fluorescence will, we hope, reduce the incidence of interference and background fluorescence. Apparatus at present under construction should meet this need.

Oestriol has presented a special problem of its own. In the chromatography of a number of samples of oestriol from different sources, we have consistently found that the first eluate fraction contains a highly fluorogenic substance, presumably not oestriol. The main fraction of the steroid is eluted in the expected place and the typical pink colour due to oestriol is observed when a sufficiently large quantity of the dried compound is heated with phosphoric acid. On the other hand, the first fraction gives a fluorescence and colour more like that of oestrone and oestradiol when similarly treated. For this reason, we doubt the validity of all our oestriol measurements. This problem still requires elucidation.

The clinical value of oestrogen determinations will inevitably be limited until adequate methods for blood oestrogens have become available. The provision of these might well be regarded as the ultimate requirement in this field.

REFERENCES

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Finkelstein, M. [1953]. *Acta endocr., Copenhagen*, 10, 149.

Oestrogens in urine during the menstrual cycle (μg/24 hr) Comparison of the methods of Brown and Bauld

Subject	Day of cycle	Brown			Bauld		
		Oestriol	Oestrone	Oestradiol-17β	Oestriol	Oestrone	Oestradiol-17β
W.M.	16	21.6	8.4	6.0	20.5	11.4	6.0
L.R.	12	10.0	9.6	3.6	6.5	8.0	3.4
E.M.	15	18.6	20.0	7.8	19.0	21.0	6.0
D.P.	20	9.8	18.8	6.2	11.5	16.0	4.5
S.A.	9	10.4	11.4	3.9	11.5	9.8	1.5
S.T.	22	12.7	13.2	3.8	12.7	17.0	3.8

I was very interested in Dr Boscott's paper. It was good to learn that someone has at last commenced a thorough study of the nature of the phenols and phenolic acids in urine. Such a study will be interesting in itself; it may also be of great importance to those concerned in the determination of urinary oestrogens, since some of these substances may be ones which interfere in the final determinations of oestrogens by the Kober or the fluorescence methods. However, I venture to suggest to Dr Boscott that he should not be content with paper chromatographic methods. My view is that while these methods are very valuable indeed for preliminary work, they are no substitute for isolation and identification by the classical methods of organic chemistry.

Dr Boscott raised the question of whether we should be satisfied with determinations of oestriol, oestrone and oestradiol, and the same question arose in Dr Swyer's paper as well as in the discussions. I think we should not be satisfied. There are various indications, the ones we heard this morning, and there are many more in the literature, that there are oestrogens—or rather Kober chromogens—in urine other than these three. These additional oestrogens must be isolated and identified and quantitative methods will also have to be worked out for them.

Dr Boscott also referred in his paper to the Smiths' zinc-HCl method for hydrolysing urine. There is no doubt that in this procedure there occurs a reduction of oestrone to oestradiol and to 3-hydroxyoestratriene. Possibly there may be also some inhibition of oxidative destruction *of oestrogens during hydrolysis, and I believe that Dr Bauld might* say that the zinc-HCl treatment may eliminate some of the substances which interfere in the Kober reaction In view of the complicated nature of what goes on in this procedure I believe that we would be well advised to forget about it for routine determination of oestrogens in urine.

GENERAL DISCUSSION

(MORNING SESSION)

Prof Marrian. I want to start my remarks by saying something about the first two papers in the programme—those of Drs Brown and Bauld. Dr Brown was kind enough to mention my name in connection with the researches which he and Dr Bauld have been carrying out, but I want to make it clear that my own part in this work was actually a very small one. Just over four years ago I asked them to try to develop a convenient and accurate method for the determination of urinary oestrogens which would be sensitive enough to be applied to samples of urine collected during the menstrual cycle. Apart from making this initial request I have contributed little if anything to their work.

My opinion of their work, which is perhaps somewhat biased, is that they have both achieved something very well worth while. Working independently, but nevertheless helping one another all the time, they have each devised a workable and convenient method for the reasonably accurate determination of as little as 5 μg each of oestriol, oestrone and oestradiol in a 24 hr urine sample. Either of these methods can be used as a routine procedure for the daily determination of oestrogens throughout the menstrual cycle

Dr Brown was very cautious about the significance of the values which he and Dr Bauld obtained for the oestrogen excretion in men. However, there is considerable evidence that the substances which they were measuring in male urines were in all probability oestriol, oestrone and oestradiol, and their recovery experiments suggest that they were determining these with tolerable accuracy I think, therefore, that Dr Brown's remarks on this matter may have been somewhat over-cautious

Both Dr Brown and Dr Bauld had misgivings about using the Allen correction formula for the elimination from their readings of the absorption due to the considerable 'non-specific' brown colour developed in the Kober reaction with extracts from urines of low oestrogen content I shared their misgivings, but my present view, based on their recovery experiments, is that what may appear to some as a misuse of this colour correction is in practice justifiable.

In the final stages of their work Drs Brown and Bauld carried out a number of determinations by their different methods on the same urine specimens, and with their permission I am showing you some of the results which they obtained (see Table, p. 49). In a few cases the agreement between the results obtained by the two methods, is not perfect, but in the majority it is amazingly good, considering the minute amounts of oestrogen which were being determined

I think that Dr Brown's results on the menstrual cycle are very interesting indeed Previous workers have reported a few studies on oestrogen excretion throughout the cycle in which methods of rather dubious accuracy were used, but I believe that this is the first time that a considerable number of different cases have been studied by means of a method of proved accuracy. I am eagerly looking forward to the time when Dr Brown is able to study oestrogen excretion in menstrual abnormalities

My second point concerns the Kober reaction. I am very surprised to hear this coming from Edinburgh, because I thought that this reaction had been well and truly interred many years ago. I think it is a pity that after such precise and meticulous work in chasing 1 μg through a tremendous flow sheet with excellent recovery, one should wreck oneself on a reaction like this. May I ask—and this may have already been tried—whether in the final methylation stage, after the oestrogens have been separated and purified, it would not be possible to use a labelled carbon, and so increase the sensitivity of detection by a factor of ten or more? If radioactive methyl sulphate is unsuitable, acetic anhydride might be used to give just that little extra sensitivity to avoid the Kober reaction.

Thirdly, I want to mention my personal, highly encouraging experience with gradient elution, particularly in ethanol-benzene on alumina. I find that this sharpens the separations considerably. It may also help to get rid of some of the objectionable chromogens that can interfere in the Kober reaction.

Dr Reiss. Halkerston and Stich in our laboratory have had extensive experience with extraction of the sulphatase and glycuronidase from these molluscs, and so far have only used them for the determination of ketosteroids. The results are very encouraging. One gets about 30% more ketosteroids by hydrolysis. And I was, therefore, very pleased when Dr Kellie mentioned that he distrusts the very intensive acid hydrolysis of urines in oestrogen determinations. So far, we have investigated only one urine with these mollusc extracts, and were surprised to get far more oestrogens after this hydrolysis than after ordinary acid hydrolysis, but that might have been just an accident.

Dr Rumney: In Prof. Zondek's laboratory in Jerusalem 2 or 3 years ago oestriol was determined in two normal menstrual cycles by the method of Finkelstein, and the results agreed very well with the results reported here.

One observation rather interested us: the increase in basal temperature occurred at a time when the oestrogen value was the lowest for the whole cycle, one day later oestrogen increased

About the question of fluorimetry and the Kober method, I think it is significant that the method reported by Dr Swyer employs 85% phosphoric acid and not sulphuric acid. This is very important because the American workers got into serious difficulties with sulphuric acid. The reaction with phosphoric acid is much more specific, and it has very great advantages over sulphuric acid. By taking 5 ml of urine and employing the Hilger fluorimeter in the final determination, one can get down to 0·01 or even 0·005 μg of oestrone.

May I also add one word about the question of enzymic hydrolysis I know that Prof. Marrian has strong views about this But it appears to me that to use acid and then to spend many hours removing products formed by the acid hydrolysis wastes considerable time. If an enzymic method can be utilized it would be very encouraging.

Dr Boscott I would like to champion the cause of paper chromatography, as was done by Bush at a recent Laurentian Conference In the paper chromatograms done by us we have used five tests, each test gives different colours or different coloured fluorescences. I might add that our I.C I reagent gives colours of azo-dyes ranging through the whole

There are a number of points in the paper by Dr Braunsberg, Miss Stern and Dr Swyer on which I would like to comment. If I understand the procedure used correctly, one of the early stages in the purification process is the extraction of the oestrogens with N-NaOH from ether solution. This process would result in quantitative extraction of oestriol, but there would be very big losses of oestrone and quite appreciable losses of oestradiol

These authors must be congratulated on developing an excellent single-column method for separating and purifying the three oestrogens in urine extracts which, I believe, will be very valuable indeed. I also think that their work on the technical side of fluorimetry is of great fundamental importance.

I noticed that Dr Swyer referred to the peak of fluorogenic material which immediately preceded the oestriol peak on their chromatograms as being due to 'non-specific' fluorogenic substances. It seems to me that this material may well be another oestrogen or another fluorogenic substance related to the oestrogens. In this connection I should mention that several workers in the United States have noted the presence of other fluorogenic substances in urine extracts in addition to oestriol, oestrone and oestradiol, while both Drs Brown and Bauld have detected the presence of a fourth Kober chromogen

I think at this point I should say something about the relative methods of fluorescence and Kober methods for the final determination of the oestrogens The former method has the great advantage over the latter in being considerably more sensitive. However, I know that it is the view of Drs Brown and Bauld that the Kober reaction is less prone to interference by trace impurities in solvents and by 'muck' in the urine extracts, and for this reason they decided to use it in preference to the fluorescence method It would be valuable if we could hear the views of others on this important point.

Dr Kellie First of all, I would like to add my congratulations to Dr Brown and Dr Bauld for their excellent work. Only those of us who have tried to process 24-hr urine samples over a course of three menstrual cycles know the tremendous amount of work that goes into getting results which you can present in a matter of a few seconds.

There are, however, a few points I would like to raise I am not experienced in the oestrogen field, but I would like to champion a very lowly animal, the marine mollusc. There are two marine molluscs which exist in abundance on the shores of England—the limpet and the periwinkle Drs Dodgson and Spencer have shown that these two molluscs are a very potent source of aryl sulphatase. By a relatively simple process one can get aryl sulphatase activity of the order of 4×10^6u /g. The preparation is also a very good source of β-glycuronidase, with an activity of the order of 700,000 u /g, which is vastly greater than one can get from any current American source It is, moreover, very cheap With regard to the use of aryl sulphatase, I notice that in the papers which have been read, everyone rather guiltily says, 'We hydrolysed the conjugates by boiling with 15% acid for 1 hr'. I suggest that this is very violent, and perhaps unwise, as there is such an excellent source of aryl sulphatase It may well be that, by using enzyme hydrolysis instead of acid hydrolysis, by the time one gets through to apply the Kober reaction, some of the troubles will disappear; perhaps there won't be such a high blank.

illusion in thinking that enzymic hydrolysis will give us pure extracts, because the main source of chromogens seems to be phenolic material, which would also be formed by enzymic hydrolysis

The final point: on gradient elution. This is unquestionably going to be the answer. If and when other oestrogens are isolated, and identified in the urinary extracts, obviously there will be a limit to how many columns you can have It seems to us that the method which Prof. Morris has developed, for gradient elution on a single partition chromatogram of the three known oestrogens, might well be advanced to the point to cover any other related compounds that we might find

Dr Boscott One of the points that I think should be looked into is the recovery of oestrogens from urines relatively rich in protein and carbohydrate, because here we have a lot of humin formation, and one does not know what the intermediates are in this humin formation. Dr Bauld has shown that aldehydes will interfere in the Kober reaction. Is it possible that furfural arising from the reaction of acid with carbohydrate during acid hydrolysis may possibly attack the oestrogens in these dirty urines? One recalls the zinc and hydrochloric acid values of toxaemic pregnancies being greatly increased, compared with ordinary hydrolysis. Is this due to the destruction of compounds arising during acid hydrolysis which would tend to destroy oestrogens, for example the sugar and protein present in toxaemia of pregnancy urines? I think these points deserve further investigation.

Dr Hannelore Braunsberg First I would like to thank Prof Marrian for his kind reception of our competitive efforts I was rather surprised, though, to hear that normal caustic soda is not supposed to extract all the oestrogens. I would be grateful for an indication of the sort of losses we may have incurred.

Secondly, I quite agree that what Dr Swyer called the 'non-specific fluorescence' coming out before the oestrone and then again before the oestriol is interesting and may have considerable significance. But I am sure it is due to a mixture of a lot of components, because it comes with the very front of the solvent, and it would need further purification and separation into various compounds

Could we perhaps join hands in this effort with the Edinburgh group? We are still rather in the dark about the validity of our results, and it would be very useful to us if we could do some fluorimetric determinations on the same samples as done by Dr Bauld and Dr Brown, and see whether we can get answers in the same region

I am sure we will find a lot of the purification steps of the Edinburgh group very useful—although our problem is really quite different. We eliminate perhaps different compounds, because they interfere with the fluorimetric measurement, and not so much because they produce a colour—we are not worried about any colours, because we use such small fractions—and I am not sure how many of your steps were just to remove colour.

I would also like to say a word in defence of fluorimetric methods. First of all, I was rather put off the Kober reaction when I read all the work done by Bandow in Germany in the 1930s His work followed on Kober's first publication, I think, and he investigated a multitude of organic compounds of all kinds, including steroids, and found that

of the spectrum, from yellow right through to mauve. By means of these relatively specific colour and fluorescence tests I think we are pretty certain of the compounds which we have identified only by paper chromatography, but we have also isolated quite a number of compounds I described.

Dr Brown: One of the primary aims in developing our method was to produce something which could be used routinely. The present method is not difficult and four of us do forty to fifty determinations a week, and have been doing so for the last 12 months. It was partly for this reason that we preferred acid hydrolysis to enzymic hydrolysis, it is easier to fit into a routine method and is free from the uncertainties of present enzymic methods.

As for the fluorescence versus the Kober reaction, I spent about 9 months on the fluorescence method at the same time as I was working on the Kober reaction, and the reason for choosing the latter was that it was the first to yield satisfactory results with urine extracts.

Dr Bauld: I would like to comment on some of the points raised by Dr Kellie. I think it is about time we changed the name of the Kober reaction It has been in existence now since 1931, and until 2 years ago the initial thing that happened when anyone worked with the Kober reaction was that they modified it. In this period the Kober reaction got a well-deserved bad name, because in each laboratory there was an art of conducting Kober assays—only one person could do it, and so on. I have spent over a year on Dr Brown's final modification and have found that a wide variety of factors—from the make of sulphuric acid to the size of tubes used—affects it But after the changes resulting from the year's work, we are confident of the stability and reproducibility of the quinol-aqueous sulphuric acid reaction As far as sensitivity is concerned, it is as sensitive as any other colour reaction in the literature, though much less sensitive than the fluorescence methods However, I think that we may be confusing sensitivity of fluorescence and sensitivity of fluorimetric measurement. There seems to be no end to it· you just put another valve in the photoelectric multiplier and then measure not 0 05 but 0 01 μg How long this can go on and how real it is I don't know

The second thing I would like to say is in connection with the acid hydrolysis. We may have appeared to be a bit guilty every time we say we boil this with acid for an hour. The reason we seem guilty is not because we have not done a great deal of investigation of acid hydrolysis, both Dr Brown and I have over the last 4 years looked into possible sources of error during the acid hydrolysis. My contention is that the apparent destruction by acid hydrolysis is not due to destruction of the oestrogens during hydrolysis, but to modification of the urine residues which at later stages in the procedure bring about a *destruction of oestrogens* In other words, I think the main effect of variations in the acid hydrolysis is on the background material, and hence on substances which go into ether extract and can destroy oestrogens subsequently during the purification procedure Our work with the final method so far gives no clear indication of a considerable destruction of oestrogens during acid hydrolysis.

I have had little experience myself with enzymic hydrolysis; we tried to avoid it, as Dr Brown said, because we wanted to have a routine method But we may be under an

I rather think that Dr Boscott misunderstood my remarks about paper chromatography. I have nothing against this—on the contrary I believe that it is one of the most valuable new laboratory techniques which have been devised in recent years, and we make very full use of it in Edinburgh. However, I reaffirm my belief that it is nevertheless not an adequate substitute for isolation and characterization by the classical methods, where these can be employed.

I think Miss Stern mentioned that she and her co-workers hydrolyse urine by boiling for 20 min with 15 vols.% of concentrated HCl. In our experience, based on many experiments carried out over the past 8 years, complete hydrolysis of the conjugated oestrogens in urine requires not less than 40 min boiling with this concentration of HCl, and in order to be quite safe we extend this minimum time to 60 min.

a very large number gave colours with sulphuric acid, and fluorescence in some cases, but not so many. He did not investigate phosphoric acid in detail. We know from Dr Boscott's work that a lot of substances will fluoresce, and I should be grateful for an indication from Dr Boscott what pathological states are likely to give us trouble and also, from the nutritional point of view, what kinds of things our patients should avoid before their urines are collected.

Miss Stern. I should like to emphasize that the fluorescence method is something like 100 times more sensitive than the Kober, not 10.

Secondly, we are a little ashamed of boiling our urines with such strong acid—for 20 min, by the way, and not for 1 hr. We have tried some very preliminary experiments with Mr Hines on some enzyme hydrolyses, which were encouraging We have also done preliminary experiments on boiling some pure oestrogens with acid, the same strength as we use for urine (15 vol. HCl/100 vol. H_2O) and then extracting with ether, without any extraction from ether with soda, but we still find quite big losses.

Prof. Marrian: First of all I must comment with some vigour on Dr Kellie's remarks about the Kober reaction This reaction has never been 'interred' by those who know how to carry it out properly, and it would seem to me that Dr Kellie has been misinformed There is no satisfactory evidence to indicate that the Kober reaction is non-specific, on the contrary the available evidence shows that if it is properly carried out it is highly specific for the naturally occurring oestrogens

The question of acid hydrolysis of urine *versus* enzymic hydrolysis was raised in the discussion, and I must make two comments about this. First of all I would say that we in Edinburgh are by no means ignoring the possibility that there may be destruction of oestrogens during acid hydrolysis, and I would remind you that Cohen and I in 1935 were the first workers to suggest that such a destruction might occur. However, I think the present view of Drs Brown and Bauld, which is admittedly based on insufficient evidence, is that this destruction may not be as serious as Cohen and I and others originally thought. Secondly, in reply to the suggestion made in the discussion that I am opposed to enzymic hydrolysis, I would remind you that as long ago as 1937 I stated (Cold Spring Harbor Symposia) that in my opinion the ultimate solution to the problem of the hydrolysis of the conjugated oestrogens would be the use of enzymes However, I feel rather strongly that at the present time the methods of enzymic hydrolysis have not been sufficiently worked out to justify their use in the routine determination of urinary oestrogens, and for that reason we think it wiser in the meantime to continue to use acid hydrolysis

In the same connection Dr Bauld raised an important point which I must underline and enlarge upon In his experience the urinary substances which interfere in the Kober reaction are not so much pigments formed during acid hydrolysis but phenolic substances which give rise to pigments by oxidation in the subsequent purification of the extracts Since such phenolic substances might well be liberated from conjugates by enzymic hydrolysis, it is possible that the latter procedure might not be so advantageous from the point of view of purity of the final fractions as many people suppose.

'conjugated' oestrogens as alcohol-soluble, but insoluble in ether and 'protein-bound' oestrogens as alcohol-insoluble and ether-insoluble. 'Conjugated' oestrogens are liberated by acid hydrolysis, 'protein-bound' oestrogens by alkali hydrolysis. The extracts are purified by solvent partition, using the scheme developed by Engel, Slaunwhite, Carter & Nathanson [1950]. The individual oestrogens in each fraction are separated by countercurrent distribution and estimated by fluorimetry.

The fluorimetric technique employed is a one-stage procedure. Alcoholic solutions of the oestrogens are heated with 88% (v/v) sulphuric acid, chilled, and the resulting fluorescence is read in a Coleman fluorimeter, Model 12 B, using a lamp filter transmitting at 4360 Å and a photocell filter with a maximal transmission at 5250 Å (Baird interference filter). Particulars of the technique are described elsewhere [Diczfalusy, 1953]. Since the fluorescence intensities of the individual oestrogens are different, it is necessary to run on each occasion standardization curves with all three oestrogens.

The separation and simultaneous characterization of the individual oestrogens is achieved by a discontinuous countercurrent procedure, as suggested by Engel [1950]. Essentially, the procedure of countercurrent distribution is based on a systematization of simultaneous multiple extractions, solutes being partitioned between series of immiscible solvent phases in separate contacting tubes in a simple countercurrent order. Since essential equilibrium is attained at each step, the results are particularly adaptable to exact mathematical interpretation. The countercurrent effect obtained by a discontinuous extraction process can be illustrated by a simple example:

[illegible] ontaining equal volumes of two [illegible] and *c* (this will be the mobile [illegible] l be the stationary phase), and start a distribution of 80 parts of a hypothetical substance, which is assumed to be exactly equally soluble in both phases. The substance is partitioned between *a* and *A*, and when equilibrium is attained, there are forty parts in the upper as well as in the lower phase, as indicated in Step 1 in Fig. 2.

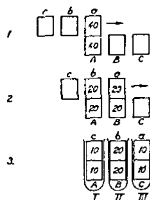


Fig. 2. Schematic representation of discontinuous countercurrent distribution of a solute whose partition coefficient is unity, in a system consisting of two transfers.

CHARACTERIZATION OF THE OESTROGENS IN HUMAN SEMEN BY COUNTERCURRENT DISTRIBUTION AND FLUORIMETRIC ANALYSIS

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SUMMARY

An attempt was made to characterize the oestrogen pattern of human semen, using solvent partition, countercurrent distribution and fluorimetric analysis.

The data obtained indicate that at least three natural oestrogens, oestrone, oestradiol-17β and oestriol are present in pooled human semen

The bulk of the seminal oestrogens was found to be present in the 'free' form

During the past decade some data have been reported in the literature indicating that human semen may possess oestrogenic activity [Green-Armytage, Silberstein & Wechtel, 1947; Rusfeldt, 1948, McCullagh & Schaffenburg, 1951]. On the other hand, negative findings have also been recorded [Bacsich, Sharman & Wyburn, 1945] We decided to reinvestigate this problem and therefore collected over the last 4 years approximately half a gallon of human semen.

The extraction procedure used in our laboratory, which was originally worked out for the extraction of placental oestrogens [Diczfalusy, 1953] is shown schematically in Fig. 1. Thus 'free' oestrogens are characterized as alcohol-soluble and ether-soluble,

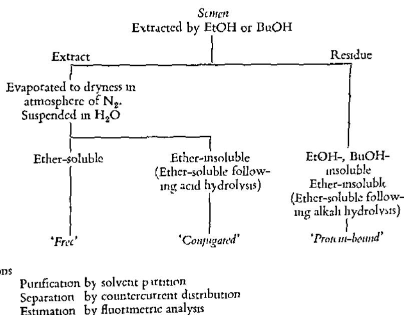


Fig 1 Schematic representation of the procedure employed for the extraction of oestrogens from human semen

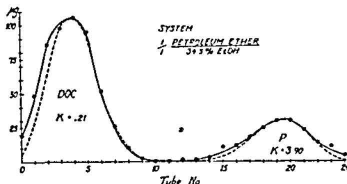


Fig 4 Complete separation of deoxycorticosterone (DOC) from progesterone (P) in a 24-transfer countercurrent distribution In upper layer petroleum ether In lower layer 34 5% (v/v) ethanol *K* is the partition coefficient for each compound calculated from the experimental curves according to Williamson & Craig [1947] For all distribution curves solid line experimental, broken line theoretical

Generally speaking, a satisfactory separation of two substances in a 24-transfer distribution can be expected if the ratio of the greater partition coefficient to the lower (the separation factor) is 3 or more. (In the example shown in Fig. 4, the separation factor is 18 6). In a distribution consisting of 100 transfers, a ratio of 1 2 is already sufficient to obtain a certain degree of separation.

The system most often used in our laboratory for the separation of natural oestrogens was devised by Engel *et al* [1950] It consists of equal volumes of 50% methanol and carbon tetrachloride. A 25-tube all glass machine, manufactured according to the specifications of Craig & Post [1949] is used. At the end of the distribution the contents of each tube are evaporated to dryness in an atmosphere of nitrogen and at a reduced pressure, the residue is taken up in ethanol and duplicate aliquots are analyzed by fluorimetry.

When crystalline oestrone, oestradiol-17β and oestriol were distributed in the system described by Engel *et al.* [1950], the pattern shown in Fig 5B was obtained; whereas the ethanol extract of 1000 ml. human semen corresponding to the 'free' estrogens gave a very similar pattern, as it would appear from Fig. 5A

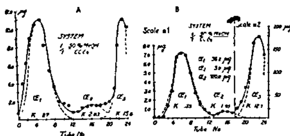


Fig 5 Countercurrent distribution of the 'free' oestrogens obtained in an ethanol extract of pooled human semen (Fig 5A) and of crystalline oestrogens (Fig 5B) In upper layer 50% methanol, in lower layer carbon tetrachloride OE_1 is oestrone, OE_2 is oestradiol-17β, and OE_3 is oestriol

The lighter phase is then shifted across the heavier one in such a way that in the next step *a* is extracted by *B* and *A* by *b*. The distribution of the substance will be now: twenty parts in *a*, twenty parts in *B*, and similarly twenty parts in *b* as well as in *A* (step 2 in Fig. 2). The mobile phase is shifted again, so that the twenty parts contained in *a* are partitioned between *a* and *C*; this operation will give ten parts in each phase *B* as well as *b* contained twenty units; in this tube the distribution remains unchanged, whereas the fraction left in *A* (20 units) will equally distribute itself between *A* and *c* (step 3 in Fig. 2) *In this way a countercurrent distribution* has been carried out in a system consisting of three tubes The binomial character of the distribution can be illustrated graphically with the amount of solute contained in each tube as ordinate and the tube number as abscissa, as shown in Fig. 3a.

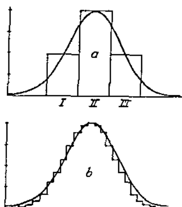


Fig 3 Effect of increasing number of transfers on relation of binomial distribution to normal curve

If countercurrent distribution of the same substance is carried out in twenty-four or more tubes instead of three, a similar pattern will be obtained, the binomial distribution approaching more and more closely a normal curve (Fig. 3b)

At this point an important term must be introduced, namely the *partition coefficient or* distribution constant (*K*), defined as the ratio of the solute in the upper phase to that in the lower phase (assuming equal volumes) In the above example it was assumed that *the substance was exactly equally soluble in both phases*, i e it had a partition coefficient of unity. However, if the substance in question is more soluble in the stationary phase than in the mobile phase, more and more solute is retained in the lower phase so that the substance will accumulate in the first tubes of the distribution. On the other hand, if [illegible] in the upper phase than in the lower phase, it will accumu-

it of the

two or

more solutes may be more or less completely separated in this way, provided their partition coefficients differ enough from each other A complete separation of deoxycorticosterone from progesterone in a system described previously [Diczfalusy, 1952] is shown in Fig. 4.

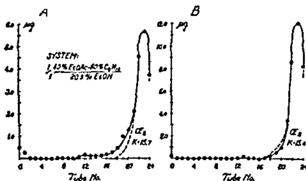


Fig 7 Countercurrent distribution of the contents of Tubes 12-16 of Figs 5A and 6 combined (Fig 7A) and of crystalline oestradiol-17β (Fig 7B) Upper layer 40% ethyl acetate, 60% hexane; lower layer 20 5% ethanol OE₂ is oestradiol-17β

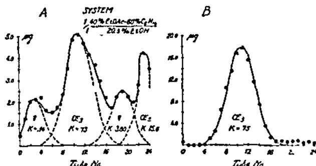


Fig 8 Countercurrent distribution of the contents of Tubes 17-24 of Figs 5 and 6 combined (Fig 8A) and of crystalline oestriol (Fig 8B) in the solvent system shown in Fig 7 OE₂ is oestradiol-17β, and OE₃ is oestriol

previously and run in the Craig machine, the distribution pattern obtained did not provide any clear-cut evidence for the presence of detectable amounts of oestrogen. Thus, although the presence of some oestrogenic material in the acid-hydrolysed fractions could not be excluded, the concentration of 'conjugated' oestrogens in the extracts investigated was assumed to be rather low. This belief was further strengthened by the negative results obtained in a screening test, in which threequarters of the 'conjugated' oestrogen fraction obtained following extraction by butanol was injected into five oestrone-primed spayed mice Since in four animals out of five negative vaginal smears were obtained, it would appear reasonable to assume that the concentration of 'conjugated' oestrogens in the extracts investigated was very low indeed.

On the other hand, when the alcohol-insoluble material was submitted to alkali hydrolysis, extracted and partitioned as described above, the distribution pattern obtained following countercurrent distribution revealed the presence of both oestrone and oestriol, although in rather small amounts (8 9 μg oestrone and 4 6 μg oestriol/1000 ml. semen). The presence of oestrogens in this fraction is in accordance with previous experiments on placental tissue and blood [Diczfalusy, 1953], in which alkali hydrolysis

According to the pattern shown in Fig. 5A, 1000 ml. semen would contain approximately 60 μg of oestrone, 10 μg of oestradiol-17β and 30 μg of oestriol.

When in another experiment 1700 ml. semen was extracted by butanol instead of ethanol, and the 'free' oestrogens distributed in the same system, the pattern shown in Fig. 6 was obtained.

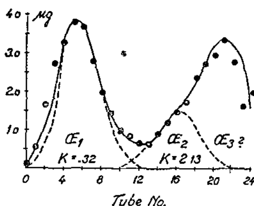


Fig. 6 Countercurrent distribution of the 'free' oestrogens obtained in a butanol extract of human semen Solvent system as in Fig. 5 OE_1 is oestrone, OE_2 is oestradiol-17β

Fig. 6 seems to indicate the presence of approximately 20 μg of oestrone and probably that of some oestradiol-17β (approx 9 μg), whereas the presence of the -triol cannot be ascertained from this distribution. It is of considerable interest to note the less favourable yield of oestrogen following extraction by butanol

Further evidence for identity of the material with partition coefficients K = 2 03 (Fig. 5A) and K = 2.13 (Fig. 6) was obtained by combining the contents of tubes 12-16 from both experiments and carrying out a second countercurrent distribution in a different solvent system. As pointed out by Engel [1950] this procedure may be compared with a mixed melting point determination, if the partition coefficients of the extracted material and that of an authentic sample of the crystalline compound agree in two different solvent systems, this is very suggestive of identity of substance, whereas a discrepancy of K-values in the second solvent system—like a depression in melting point— proves that the two substances are not identical. When the combined material from tubes 12-16 (Figs 5A and 6) was distributed in another solvent system consisting of ethyl acetate-hexane as upper phase and aqueous ethanol as lower phase, and the calculated partition coefficient was compared with that of crystalline oestradiol-17β distributed in the same system, a close agreement of partition coefficients was found, indicating that the material is, in fact, identical with oestradiol-17β (Fig. 7)

Again, when the contents of tubes 17-24 from the two experiments (see Figs. 5A and 6) were combined and this material was distributed in the system shown in Fig. 7, the calculated partition coefficient (K = 0.78) was found to be very nearly identical with that of crystalline oestriol (K = 0.75), as it would appear from Fig. 8

When, following the removal of 'free' oestrogens, the residual seminal extracts were hydrolysed in 15% (v/v) hydrochloric acid for 30 min, extracted by ether, purified as

Dr Mann: It would be very interesting to repeat these very beautiful experiments on spermatozoa and seminal plasma separately.

Dr Diczfalusy: It would take years.

Dr Mann: In man, unlike the hog, the bulk of the semen is composed of seminal plasma. So, your oestrogen may have nothing to do with the spermatozoa; it may be all in the accessory fluids.

Dr Diczfalusy: I don't think so McCullagh & Schaffenburg [1951] have separated them, and state that the oestrogenic activity is due to the spermatozoa. I have no experimental data.

Dr Mann: It would be very important to settle that point.

of ethanol- and butanol-extracted material invariably liberated a small fraction of oestrogen. Whether the presence of this 'protein-bound' oestrogen fraction depends on adsorption, incomplete extraction, solubility of oestrogen in protein solutions, or possibly on a chemical bond, remains to be established.

Further experimental details of the work described in outline in this paper are reported elsewhere [Diczfalusy, 1954].

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DISCUSSION

Miss Stern: I understand that the oestrogens you find in human semen are largely in the free form. Was there evidence of any protein-bound oestrogens?

Dr Diczfalusy It depends on the definition of the term. If you accept the term 'protein-bound' oestrogens as indicating a chemical bond, we have never detected them. We simply cannot account for this fraction, so it is denoted very tentatively as 'protein-bound oestrogens'.

Miss Stern I was thinking of 'protein-bound' more as Samuels *et al.* did in their recent paper in the J biol. Chem. [1954 206, 751] and found it loosely bound They used albumen in their experiments

Dr Diczfalusy It might depend on failure of extraction techniques. I think we must be very cautious on this point.

Dr Hannelore Braunsberg Were you quite satisfied with the caustic soda treatment? I presume in the extraction of protein-bound material you heat the fluid with caustic soda

Dr Diczfalusy. No, we leave it at room temperature for 24 hr with 5% NaOH, it is added in equal volumes, so you get a final concentration of 2 5% NaOH.

Dr Hannelore Braunsberg. Does no destruction of oestrogens occur with this treatment?

Dr Diczfalusy We tried to do the same with crystalline compounds, and found no destruction, even when heating the crystalline oestrogens, I think Dr Bauld can confirm this. On the other hand, I have not tried oestrogens added to extracts of body fluids

Dr Hannelore Braunsberg· In Prof Samuels' study, which Miss Stern just mentioned, they allowed the protein-binding to take place, and then just shook up the protein-bound steroids (not just oestrogens, but all sorts of steroids) with ether and got them all out again.

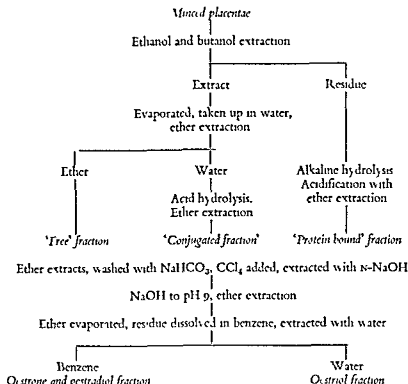


Fig 1. Extraction of 'free', 'conjugated' and 'protein-bound' oestrogens from tissue, and their purification and separation into oestrone-oestradiol and oestriol fractions

The solvent system for oestrone and oestradiol was made from equal volumes of light petroleum (boiling point range 100–120°) and methanol, and for oestriol, 2 vol. of benzene to 1 vol. of methanol and 1 vol. of water. The solvent systems have been so arranged that the runs may take place overnight, the *Rf* values in the respective systems are oestrone, 0.2; oestradiol-17β, 0 07; and oestriol, 0 13

As a simple and sensitive method for the detection of the oestrogens on paper was not available, Folin and Ciocalteu's reagent [Folin & Ciocalteu, 1927] was investigated and found to be ideal for this purpose, this is a reagent which is used in routine biochemistry for the estimation of phenol The paper was lightly sprayed with the reagent diluted with water, and then placed in a tank, the atmosphere of which was saturated with ammonia vapour. The oestrogens show up as blue spots on a pale blue background with a detection limit of 5 μg/sq cm. This reaction may also be carried out quantitatively in solution for the colorimetric estimation of pure oestrogens, but as the colour is produced with all phenols, it is not suitable for use with extracts, in which phenolic impurities are invariably present.

The amount of the oestrogen in the spots was estimated by fluorimetry and, as a prior spraying with Folin & Ciocalteu's reagent would interfere, the positions of the spots were found by means of parallel runs with smaller amounts of similar extract and of pure oestrogens The smallest area of paper ensuring removal of all oestrogen in the

THE ESTIMATION, RECOVERY AND NORMAL LEVELS OF OESTRIOL, OESTRONE AND OESTRADIOL IN HUMAN FULL-TERM PLACENTAE

By F. L. MITCHELL

From the Jessop Hospital for Women, Sheffield 3, and the Medical Research Council Unit for Research in Cell Metabolism, The University, Sheffield 10

While much work has been carried out in recent years on the clinical estimation of oestrone, oestradiol and oestriol in urine, little work has been reported on the estimation and detection of the oestrogens in tissue. Diczfalusy early last year [Diczfalusy, 1953] reported the use of the countercurrent method of Engel, Slaunwhite, Carter & Nathanson [1950] for the separation and purification of endogenous oestrogens in placental tissue, but as recently as 1952 Goldzieher & Roberts [1952] had to use over 8 kg of tissue and rely on only a chemical separation to identify the oestrogen in human testes.

In an attempt to achieve the high degree of purification necessary for a final estimation by fluorimetry, a total of six chromatographic methods of separation and one chemical method of purification were tried before the technique of paper chromatography at elevated temperature described by Bush [1952] for adrenal steroids was investigated and found to give satisfactory results. By this means 1 μg of each oestrogen may be detected and estimated in up to 200 g of placental tissue.

The tissue was minced and mixed with ethanol within 30 min of delivery; the oestrogens were extracted, separated into 'free', 'conjugated' and 'protein-bound' extracts by a method similar to that used by Diczfalusy [1953], and each extract was purified and separated into oestrone-oestradiol and oestriol fractions by the method shown in Fig. 1. This separation was found necessary because the high polarity of oestriol compared with oestrone and oestradiol entails the use of two separate solvent systems for chromatography.

If greasy material was still present in an amount which interfered with chromatography, i.e. more than approximately 1 mg per run, this was removed by washing a solution of the extract in 90% (v/v) methanol with *n*-pentane [Ryan & Engel, 1953].

All solvents were rigorously purified before use and operations above room temperature were carried out under nitrogen. Extracts were stored at $-20°C$ when not actually in use. The final fractions were taken up in small amounts of ethanol for transferring to the 18 in. strips of Whatman No. 541 filter paper. Extracts from tissue up to 100 g may be run as one spot.

The chromatography was carried out by the descending method at a temperature of 32°, the temperature throughout the cabinet varying by not more than 0.5°. It was essential that the inner walls of the tanks should be covered with wads of filter paper dipping into the mobile and stationary phases to ensure saturation of the atmosphere with solvent vapour.

Table 1. *Comparison of the amounts of oestrogen in paper chromatogram spots when assayed by various methods*

Method of Assay	Free oestrogen (μg) per 400 g placenta Oestriol	Oestrone	Oestradiol
Visual comparison of spots	40	16	4
UV absorption with approx. corr. for impurity	41	25	4
Biological	24-60	17	Trace
Fluorimetry	40.5	13.8	5.0

To arrive at the true level of the oestrogens in the samples of placental tissue it was necessary to complete several large-scale recovery experiments. These experiments have only been carried out on the 'free' fraction. Amounts of 500 μg and 30 μg of each oestrogen were added to two similar batches of placental mince, each batch weighing approximately 200 g. The recoveries were similar for the large and the small amounts of oestrogen, and averages are shown in Table 2. Heating the tissue to 100° in nitrogen before adding the oestrogen increased the recovery in all cases, whilst heating in air increased it further.

Table 2. *The recovery of 'free' oestriol, oestrone and oestradiol added to full term human placental tissue (The pure oestrogen in ethanolic solution was added to the mince immediately after the addition of 80% ethanol for extraction)*

Expt No	No of placentae pooled	Treatment of placentae before extraction	Recovery of added 'free' oestrogen Oestriol (%)	Oestrone (%)	Oestradiol (%)
1	3	None	21.0	17.0	11.7
		Heated 25 min at 100° in N_2	52.9	24.9	19.2
2	2	None	43.4	17.2	15.4
5	2	None	18.5	4.5	15.0
		Heated 25 min at 100° in air	99.0	13.0	70.0
Aver. recovery from untreated placentae			26.7	13.5	13.7

Table 3 gives the experimental values found for the 'free' oestrogens in different placentae compared with the estimated true values after correction for the low recoveries. It will be seen that though heating the mince in air causes increased recovery (Table 3), it results in the loss of a large amount of the original oestrogen. Heating in nitrogen gives a smaller loss of endogenous oestrogen, which is counterbalanced by an increased recovery, to give an experimental value similar to that obtained for untreated placenta. The addition of thiourea to act as an antioxidant did not improve the yield.

main runs was then cut out, together with similar areas from a blank part of the chromatogram, and standard amounts of oestrogen were placed on these blank papers.

For quantitative work, the chromatography paper used was extracted with methanol for 48 hr, but, because at the end of this time methanol-soluble impurities were still extractable from the paper, each spot was extracted with the same amount of solvent for a standard time, and a special apparatus was constructed which dripped 5 ml. methanol over each paper for a period of 8 min. This ensured the extraction of all the oestrogen and the same amount of paper impurity in both tests and standards. Fluorimetric assays were carried out by the method of Diczfalusy [1953].

To prove the identity of the spots, extracts from 160 g of placental tissue were run on the chromatograms and the spots extracted. The ultraviolet absorption spectra were compared with those for pure oestrogens (Fig. 2), and a portion of the extracts were coupled with Fast Black Salt K followed by chromatography [Heftmann, 1950]. In each case well defined spots were produced, identical in all ways with spots produced by parallel runs of pure oestrogens.

The spots have been assayed by four separate methods (including bioassay) with good agreement of results (Table 1).

Recovery experiments with the three oestrogens added to the tissue mince showed that during the extraction and purification process, despite the precautions taken to purify all solvents, to reduce oxidation, and to store at a low temperature, a considerable loss of oestrogen took place. Very little loss was experienced during the chromatography and elution from the chromatogram, for when the oestrogens were added to the extract placed on the paper they were recovered as follows; oestriol 97%, oestrone 95%, and oestradiol 84%. These results also indicate that little quenching of fluorescence takes place by any tissue impurity present in the spots.

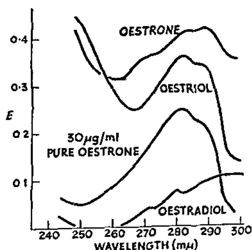


Fig. 2. Absorption spectra of oestrogen spots from 160 g placental tissue, eluted from the chromatograms and compared with pure oestrone. Absorptions measured in ethanol against extractions from similar areas of blank chromatogram paper.

Table 4. *Experimental values for the 'conjugated' and 'protein bound' oestrogens found in placentae*

Expt No.	No of placentae pooled	Treatment of placentae before extraction	'Conjugated' oestrogens isolated (μg/kg)			'Protein-bound' oestrogens isolated (μg/kg)		
			Oestriol	Oestrone	Oestradiol	Oestriol	Oestrone	Oestradiol
1	3	None	65	25	0	33	16	0
		Heated 25 min at 100° in N_2	68	23	0	31	16	0
3	1	None	70	34	14	27	27	24
5	2	None	0	8	17	0	5	10
		Heated 25 min at 100° in air	0	8	8	0	1	17
Aver content of untreated placentae			44	21	8	21	14	7
Content as found by Diczfalusy [1953]			31.4	2.5	1.5	10 8	3.4	0

Table 3 *Experimental values for the 'free' oestrogens found in placentae, with values corrected by recovery experiments (cf. Table 2)*

Expt. No.	No of placentae pooled	Treatment of placentae before extraction	'Free' oestrogen isolated (µg/kg) Oestriol	Oestrone	Oestradiol	'Free' oestrogen corrected (µg/kg) Oestriol	Oestrone	Oestradiol
1	3	None	157	108	0	748	635	0
		Heated 25 min at 100° in N_2	168	111	0	317	317	0
2	2	None	220	109	13	522	651	89
4	2	Heated 25 min at 100° in N_2+thiourea to 0 1%	288	194	10	—	—	—
		Heated 25 min at 100° in N_2	275	194	8	—	—	—
5	2	None	148	20	76	730	440	507
		Heated 25 min at 100° in air	92	19	57	93	146	82
Aver. content of untreated placentae			190	86	25	678	584	170
Content as found by Diczfalusy [1953]			125 4	46.7	3.1			

experiment oestrone gave very poor recoveries. I just wondered where we should find the cause of the discrepancy.

Dr Mitchell: I may be wrong, but wasn't Dr Engel working with enzymes?

Dr Diczfalusy: No, it was liver slices incubated.

Dr Mitchell. But here we hope the enzymes are all destroyed by the addition of alcohol before addition of the oestrogens. Also, the heating would take care of all the enzymes which were not destroyed by alcohol. We originally heated the tissues in the hope of removing proteins which interfered with partition coefficients by producing emulsions; the heating does this to a large extent. It also has this side-effect, which we thought was very interesting.

Dr Diczfalusy: Would you be interested in a joint effort to reinvestigate this problem? I have stored some early placenta (a few kg) in deep freeze at $-20°$. I could send you some and we could try to carry out simultaneous experiments

Dr Mitchell: Certainly; that would be very interesting. It is one of the drawbacks: you have worked on early placenta, and we have used full-term placenta.

Dr Hannelore Braunsberg: I am interested in your comment on the maternal blood oestrogens How did you try to measure them, and were you able to detect anything at all?

Dr Mitchell: We did them virtually by my method for placental mince. In each case we got out spots which had the same Rf values as the three oestrogens, but we did not do any biological assays to characterize them.

Dr Hannelore Braunsberg: How did you show them up? With the Folin-Ciocalteu?

Dr Mitchell: Yes.

Dr Hannelore Braunsberg: That does not detect smaller amounts than about 5 μg, does it, after running?

Dr Mitchell: No, that is true. For these runs we used large amounts for detection For the other assays, although we cannot detect the spots by the Folin-Ciocalteu reagent, by using parallel runs, you cut out where you hope the oestrogens are, and you can estimate down to about 0.1 μg fluorimetrically, even though you cannot see it.

Dr Rumney: The metabolism of natural oestrogens *in vitro* by cell-free enzymatic extracts of rat and human liver has been recently studied by us The solvent system benzene/methanol/water was found convenient for the separation of all three natural oestrogens by paper chromatography, using the Folin-Ciocalteu reagent for detection, and fluorimetry with phosphoric acid for quantitative estimation Both oestrone and oestradiol were found to be partly interconverted, but neither gave rise to oestriol with either rat or human liver extract. A phenolic metabolite more polar than oestriol was detected, and we are trying to identify this. Recently, we have also obtained the spleen from a case of Gaucher's disease. After incubation of oestrone with a homogenate of this tissue, no phenolic metabolite could be detected on the chromatogram Recovery of oestrone after incubation with boiled tissue was 85%

Dr Bauld. I would like to suggest that Fe^{+++} is the factor in Dr Mitchell's experiments which is able to destroy oestrogens in the placenta during the subsequent purification process. In many of our experiments in Edinburgh traces of iron added to the water for our blank extractions, even at room temperature, caused massive destruction of

The corrected amounts of 'free' oestrogen are between four and sixteen times more than the uncorrected values obtained by Diczfalusy [1953] which are similar to those obtained by Huffman, Thayer & Doisy [1940], except that the latter reported 38 μg of oestradiol/kg. The uncorrected values for the 'conjugated' and 'protein-bound' fractions (Table 4) are also somewhat higher than those found by Diczfalusy [1953].

To ascertain how much of the oestrogen in the tissue was due to the small amount of retained blood, the placental, maternal and foetal blood content was measured, and estimations were carried out on maternal blood. Though the sensitivity of the method was not sufficient to give accurate results on the 10 ml. volumes available, it was sufficient to show that the blood content of the placental tissue could not account for more than 5 μg/kg of each oestrogen (uncorrected), assuming that the foetal blood oestrogen is not higher than the level in maternal blood

Diczfalusy has carried out recovery experiments only on early placentae (3-5 lunar months) and has obtained recoveries of 40 to 90%. The results shown in Table 2 seem to indicate that full term placentae contain a factor which at some stage in the extraction and purification process is capable of destroying the oestrogens. From the recoveries obtained by Diczfalusy [1953] this factor does not appear to be present to the same extent in early placentae. It appears that the factor itself is partly destroyed by heating the tissue in air prior to extraction, though this procedure results in a large proportion of the oestrogens themselves also being destroyed.

To summarize briefly: a method has been developed for the estimation of the oestrogens in placental tissue by means of a preliminary extraction and chemical separation, followed by paper chromatography and fluorimetric assay. Recovery experiments using this mode of assay show that the normal methods of extraction and purification of the 'free' oestrogens when applied to placental tissue give average losses of from 73 to 86%.

Experimental details of the work described in outline in this paper have been reported elsewhere [Mitchell & Davies, 1954].

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DISCUSSION

Dr Diczfalusy: I am worried about the bad recoveries you get in full-term placenta Of course, Dr Engel noticed a very rapid inactivation or destruction or transformation of oestradiol when it was added to full-term placenta, and there was much less in early placenta. So that might be the explanation, but I am not quite convinced In our experience the best recoveries were invariably obtained with oestrone, whereas in your

THE BIOLOGICAL ASSAY OF OESTRONE OESTRADIOL IN URINE

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SUMMARY

4 Preliminary investigations of both gonadotrophin and oestrone oestradiol excretion were often diagnostic of either pituitary or ovarian failure, but single determinations of oestrone oestradiol were seldom of value

5 Oestrone oestradiol excretion in normal prepubertal girls was well below normal menstrual cycle values Seven out of eight girls with precocious puberty did not show raised excretion of oestrone oestradiol. In the eighth patient, who menstruated regularly, both gonadotrophin and oestrone oestradiol were within the normal adult range.

6 Increased amounts of oestrone oestradiol were excreted by a patient with an adrenal tumour

Six years ago, when this work began, the use of oestrogen excretion as an indication of ovarian and adrenocortical activity had been realised, but had been little investigated. Chemical methods were not then capable of measuring the very small amounts of oestrogens present in normal urine, although pregnancy urines could be more satisfactorily analysed. It was therefore necessary to use a biological method for measuring oestrogens, this, although laborious, was capable of detecting the fluctuations occurring in the normal menstrual cycle and of detecting the amounts found in the urine of children.

The Allen-Doisy vaginal smear technique is usually employed but has the disadvantage that animals respond differently to different oestrogens when these are injected subcutaneously. Emmens [1941, 1950] has described an intra-vaginal method of administration, but Biggers [1951] has shown that although oestrone, oestriol and oestradiol may have a common slope and potency under certain conditions of intra-vaginal administration, the common slope of the dose response line so obtained is too low for the method to be applied to practical problems

A two-injection Allen-Doisy technique, as described by Emmens [1939] was used for the work described below, which took place over a period of 6 years. Oestrogens were estimated in the urine of a series of normal subjects for comparison with urinary oestrogen values found in patients with various endocrine disorders.

METHOD

Specimens of urine were collected by each patient without preservative every 24 hr Acid hydrolysis was followed by extraction with ether. Oestrone and oestradiol were

oestrogens, and there is a possibility that you are dealing with iron released by haemolysis of maternal blood within the placenta This could be easily checked by seeing whether chelating agents do or do not improve your yields.

Dr Mitchell: If it was from haemoglobin, Veldhuis would not have obtained his excellent recoveries of oestrogens.

Dr Bauld Veldhuis did his recoveries on plasma, not on whole blood.

Dr Boscott: I should like to congratulate Dr Mitchell on his very excellent paper, especially in view of the very troublesome compounds one finds in tissues such as placenta. Could he tell us what his experiences are in relation to interference from phospholipids, which I have found to be very troublesome indeed in other methods of steroid extraction. Do you get any interference from phospholipids, emulsions, etc ?

Dr Mitchell: We did. We have tried several methods of purifying the oestrogens, and the manoeuvre which gave the best removal of phospholipids and the least trouble with emulsions was extracting the oestrogens from ether-CCl_4 by means of N-NaOH.

Dr Boscott: Do you not get any fatty acids carried through?

Dr Mitchell: Well, if they are carried through, there is no trouble with emulsions later on.

Dr Boscott: You do not get emulsions at the ether-CCl_4 extraction stage?

Dr Mitchell: No, with this technique there is no emulsion trouble anywhere. We had considerable trouble for 2 years with phospholipids, and it was the main reason for discarding the six chromatographic techniques which we tried. Eventually we found paper chromatography was the only answer. Of course, Dr Diczfalusy gets good results, using countercurrent distribution.

Dr Rumney: With the Folin-Ciocalteu reagent I think we can detect 1-2 μg.

Dr Mitchell We reckoned 0.5 $\mu g/cm^2$ or so; on a spot of perhaps 4 cm^2 it would be about 2 μg

ual cycles of five young
Two peaks of oestrone
ne end of the cycle at a
variable time before menstruation During a period values fell to less than 4 μg/24 hr. The range found in these cycles was 2 5–35 μg/24 hr. In some cycles the approximate time of ovulation was found by pregnanediol determination

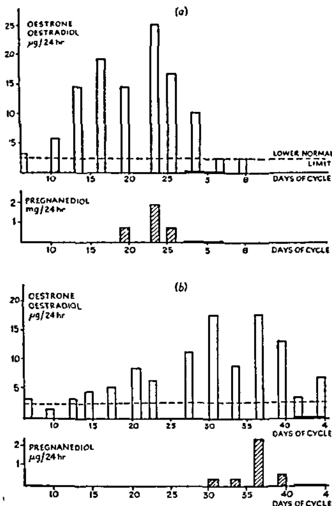


Fig 1 Oestrone oestradiol and pregnanediol excretion in the menstrual cycle of a normal woman, age 24 (a) 27-day cycle, (b) 41-day cycle

The excretion of oestrone oestradiol and pregnanediol during two cycles from one woman are shown in Fig. 1, a and b Peaks in oestrone oestradiol excretion occurred on the 11th and 5th days before menstruation, while pregnanediol was maximal at the second peak on both occasions In the second cycle, which extended to 41 days, there appeared to be a delay in ovulation.

separated by a method previously described by Edwards, Gray & Wood [1950]. The oestriol fraction was removed during the process.

Ovariectomized albino mice were used for the assay. Each mouse was given two subcutaneous injections of nut oil containing a suitable aliquot of the oestrone oestradiol extract. Vaginal smears were taken three times to cover the period of oestrous response Oestrous and pro-oestrous changes were classed as positive. Standard doses of oestrone were included with each batch of unknowns in order to check the sensitivity of the mice.

Slope of the dose response line

Slopes of the dose response lines for pure oestrone, oestradiol and oestriol solutions were calculated from the weighted means of data obtained over a long period. The slopes for oestrone and oestradiol were satisfactory, but that for oestriol was too low to permit accurate estimation A common slope for all three compounds was found to be invalid, but there was no significant difference between the slopes of oestrone and oestradiol (P = 0.8). Oestrone and oestradiol were therefore estimated together and the oestriol fraction discarded. No significant difference was found between the slope of urinary oestrone oestradiol extracts and that of pure oestrone.

Table 1. *The mean slope of dose response lines*

	Mean slope ± S.E
Oestrone	4 61 ± 0.297
Oestradiol	4.38 ± 0.796
Oestriol	1.87 ± 0.236

χ^2 Test Oestrone-oestradiol slopes $\chi^2 = 0{\cdot}13$, $P = {\cdot}8$
Oestrone-oestriol slopes $\chi^2 = 54$, $P < {\cdot}001$

For practical reasons the number of mice which could be used for each estimation was limited, only fifteen or twenty mice were used for each dose level Two dose levels were given of each standard and unknown. The theoretical limits of error using these numbers were 69-144% and 73-137% respectively.

Urinary pregnanediol was measured by the method of Sommerville, Gough & Marrian [1948]. Urinary gonadotrophin was extracted by the tannic acid precipitation method described by Levin & Tyndale [1936]. Later the kaolin adsorption method of Dekanski [1949] was substituted as the final extracts were less toxic. Gonadotrophin was assayed by a method depending on the increase in uterine weight produced in immature female rats. Later a more sensitive mouse method was used.

RESULTS

Normal menstrual cycles

The excretion of oestrone oestradiol during the normal menstrual cycle was investi-

Table 2 *Primary amenorrhoea*

Condition	Age	Oestrone oestradiol µg/24 hr	Gonadotrophin m u u /24 hr*
Turner's syndrome	29	1.0	>96
Turner's syndrome	15	4.6	>73
Diabetic dwarf	20	3.1	<6
Mild hypothyroidism	19	3.1	<20
No related complaint	21	3.8	48
" " "	18½	.8-2.8	Detected once

*Mouse uterine units

expected the oestrone oestradiol excretion was low and the gonadotrophin above normal limits. These patients have vestigial ovaries only and oestrogens were produced presumably by the adrenal cortex.

The two following cases showed a low excretion of both oestrone oestradiol and gonadotrophin. The diabetic girl, who showed slight pituitary dwarfism, subsequently took her own life. Autopsy showed that the ovaries were of normal size and the pituitary was macroscopically normal, the adrenal cortex was atrophied. Insufficient production

her only periods had been scanty and occurred at 6 months and a year before the investigations. With continued thyroid treatment, however, her general condition improved and her periods became normal The delay might have been due to late production of gonadotrophin, apparently associated with thyroid deficiency

In the last case, amenorrhoea was the only complaint. Oestrogen therapy had induced bleeding on the first occasion, but only a slight show was produced in subsequent months of treatment, this did not persist after treatment ended Gonadotrophin and oestrone oestradiol excretion, measured some months after treatment ended, were within normal limits. Her uterus had remained small and was apparently unresponsive to oestrogens.

Secondary amenorrhoea and oligomenorrhoea

Eight patients with secondary amenorrhoea were investigated In five, oestrone oestradiol estimations were made over a month or more; one investigation only was made on each of the remaining three.

In the first case, a woman aged 32, periods had been regular until she was 20, then at 3-6 monthly intervals until the last 2 yr, when they had ceased altogether. Values for both oestrone oestradiol and gonadotrophin excretion (Fig. 3a) were within normal limits for menstrual cycles, but neither showed any significant fluctuations, such as are

Oestrone oestradiol excretion in amenorrhoea

In many cases of amenorrhoea the normal relationship between pituitary and ovarian hormones has either been disturbed or has never been established owing to primary insufficiency of one or more of the hormones concerned. Low excretion of both oestrone oestradiol and gonadotrophin may be due to pituitary hypofunction, whereas low oestrone oestradiol and excessive gonadotrophin indicates ovarian hypofunction. In other cases hormone excretion is normal, but the uterine mucosa may be insensitive to oestrogens and bleeding may never be produced.

Primary amenorrhoea

Urinary oestrone oestradiol and gonadotrophin excretions were determined at intervals of 3 or 4 days for a month in two cases of primary amenorrhoea and values for both were consistently low. The results in one of these cases is shown in Fig. 2. There was no evidence of cyclical variation and such differences as do occur may be within the limits of experimental error. The investigations were made when the patient was 19 She had developed normally with the appearance of pubic hair and small breast development at 14, but her only period had been induced by a short course of stilboestrol which was not, however, taken regularly. In this case the amenorrhoea seems to be due to insufficient production of gonadotrophin, normal menstrual cycle values lying between 5 and 70 mouse uterine units (m.u.u.)/24 hr.

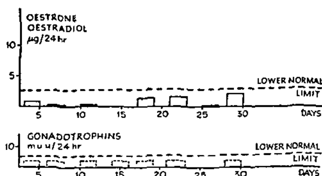


Fig 2 Oestrone oestradiol and gonadotrophin excretion in a girl with primary amenorrhoea, age 19

[illegible] excreted were measurable on one occasion. Large cyclical doses of oestrogens had been given, but bleeding was produced on only two occasions The uterus remained infantile and appeared to be unresponsive to oestrogens. Pregnanediol was detected on two occasions, but was not investigated over the month Amenorrhoea was apparently due to insufficient oestrogen production and an insensitive uterus

It is sometimes possible to differentiate between such abnormalities by a single determination of oestrone oestradiol and gonadotrophin excretion (Table 2).

The first two subjects in Table 2 were examples of Turner's syndrome. As would be

this treatment her oestrone oestradiol excretion was less than 2.5 μg/24 hr on 4 consecutive estimations, although pregnanediol was found. She later became pregnant without further treatment. Of the remaining two, one was diabetic and the other, after having amenorrhoea for 9 years, showed no response to progesterone or to ethinyl oestradiol and ethisterone.

Abnormalities in the excretion of oestrone oestradiol were found in all these women with secondary amenorrhoea. In some the amounts excreted were very low, although not always accompanied by abnormal gonadotrophin excretion, in others normal quantities of oestrone oestradiol were excreted, but with no cyclical variations and no maintained peaks of excretion. In two cases oestrogen therapy made pregnancy possible, although regular periods could not be maintained on ceasing treatment.

Irregular periods and sterility

Two patients with irregular periods and sterility were investigated. The oestrone oestradiol and gonadotrophin excretion of the first of these is shown in Fig. 3b. This woman, aged 28, had periods at irregular intervals They had occurred 12 days before and 8 days after these investigations. Normal quantities of both gonadotrophin and oestrogens were found. Gonadotrophin increased during the first part of the cycle and decreased in the second part, whereas values for oestrone oestradiol were very low during the first 26 days and then showed a sudden rise with a peak about 15 days before the next period Therefore, apart from a delay in oestrogen production, the first part of the cycle appeared normal Unfortunately the last part, when a further rise in oestrone oestradiol would have been expected, was not investigated. The time of ovulation was not known, but it could well have occurred at the first oestrone oestradiol peak. The patient was later found to have a congenital abnormality of the uterus.

In the second case, a woman aged 26, oestrone oestradiol excretion was below normal during the first part of the cycle, but a pronounced increase occurred in the latter half. Gonadotrophin excretion was consistently subnormal except at one point 4 days before the rise in oestrone oestradiol excretion. This patient had a tubal insufflation immediately following the investigations and became pregnant shortly afterwards Therefore, although her excretion of both gonadotrophin and oestrone oestradiol differed from the normal pattern, the amounts produced were sufficient for pregnancy to occur

Oestrone oestradiol excretion in normal children

Table 3 *Normal children*

Girls Age	Girls Oestrone oestradiol μg/24 hr	Boys Age	Boys Oestrone oestradiol μg/24 hr
4	.9	5	1.4
4	1 4	8	2 4
4	9	10	8
5	1 4	11	.6
8	1 1	11	7
11	3 5		
13	2 4		

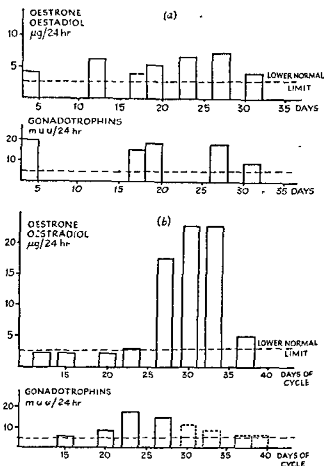


Fig 3 Oestrone oestradiol and gonadotrophin excretion in two women with (a) secondary amenorrhoea and sterility, age 32, (b) irregular periods and sterility, age 28

found in normal menstrual cycles. Her uterus and ovaries were both small, but hormones were obviously being produced by both ovaries and pituitary. Her failure to menstruate is associated with imbalance rather than insufficiency of hormones

A similar excretion of oestrone oestradiol was found in a girl of 19, who had had only 6 periods, all in the previous 2 yr Again no marked oestrone oestradiol excretion peaks were found and all the values lay between 4 and 12.1 μg/24 hr The gonadotrophin excretion was measured by the rat method which is less sensitive and accurate than the mouse method. No pregnanediol was found Like the first case she had very irregular periods, although they have since become more frequent There was both failure in ovulation and in regular production of oestrogens.

In the remaining six patients with secondary amenorrhoea, oestrone oestradiol excretion was below normal Three of these were probably prematurely menopausal, as gonadotrophin values were abnormally high. One girl of 25 had had no periods for 3 yr, except those induced by oestrogen therapy. At a time when she was not receiving

was not possible to obtain enough specimens to see if the amounts varied as they do in the normal adult cycle, but they came within the adult range.

Regular bleeding also occurred in another girl of 2½, but the values were not abnormal for her age. Laparotomy at which biopsies of both ovaries were taken showed that one contained a follicular cyst and the other a corpus luteum. A third case was receiving deep irradiation of the pituitary to inhibit the gonadotrophic stimulus. Her oestrone oestradiol excretion was normal for her age.

These results suggest that in most cases of precocious puberty oestrone oestradiol measurement is of little value as the amount excreted is not abnormal. There are, however, rare cases of granulosa cell tumour of the ovaries where a high oestrone oestradiol output would be diagnostic, but we did not have the opportunity to investigate such a case.

This method of oestrone oestradiol determination becomes very inaccurate when the small amounts excreted by children are measured; nevertheless these amounts are considerable if their small body weight is taken into account. Such quantities might be sufficient in combination with other factors to cause the changes found in precocious puberty.

Oestrone oestradiol excretion in adrenal carcinoma

Carcinoma of the adrenal cortex is sometimes accompanied by a high output of oestrogens. We were able to obtain specimens from a woman aged 40 before and after removal of an adrenal tumour. The first estimation before the operation gave the abnormally high value of 52 μg in 24 hr. The second estimation, 2 months after the operation, showed a reduced value of 7.4 μg in 24 hr, which is well within normal menstrual cycle limits. This suggests that the tumour had caused the high oestrogen output.

Table 5 *Oestrone oestradiol excretion in patients with malignant tumours*

Condition	Time of specimen	Oestrone oestradiol μg/24 hr
Adrenal carcinoma	Preoperative 1 month	52.0
	Postoperative 2 months	7 4
Granulosa cell tumour of the ovary	Postoperative 4 months	30 0
	Postoperative 5 months	23 6

Oestrone oestradiol excretion in granulosa cell tumour of the ovary

In the only case we investigated it had not been possible to obtain specimens before operation, when a large tumour, afterwards found to be a granulosa cell tumour of the ovary, was removed. Postoperative estimations were made to check the complete removal of the tumour. Values were high in specimens taken 4 and 5 months after operation, although the patient, aged 49, was menopausal, but there has been no recurrence of symptoms after 2 years.

The oestrone oestradiol excretion of normal children was measured using the whole of 24 hr urine specimens. These results are shown in Table 3 Apart from the two eldest girls, aged 11 and 13, who were probably approaching puberty, all the values for girls lay between 0.9 and 1.4 µg/24 hr which is well below the limit for the normal menstrual cycle. The oestrone oestradiol excretion in prepubertal boys was not significantly different, the range being 0 6–2 4 µg/24 hr.

Oestrone oestradiol excretion in children with precocious puberty

The oestrone oestradiol excretion of several girls with precocious puberty was measured Two of these menstruated regularly and vaginal bleeding had occurred in some of the others; all showed breast enlargement and adult female growth of pubic hair The results are shown in Table 4.

Table 4. *Precocious puberty in girls*

Age	Time of Specimen	Oestrone oestradiol µg/24 hr	Comments
2½	28th day 5th day 17th day	3.5 2.4 5 0	Menstruating regularly, gonadotrophin detected *on 22nd, 11th, 27th days of consecutive cycles*
2½	5 3.51 *17. 7.51*	1 4 *0 56*	Recurrent uterine bleeding. Advanced physical *development, follicular cyst in one ovary, corpus* luteum in the other
4	6 11.50 20. 7.51	1.6 0 6	Breast enlargement and pubic hair No menstruation, receiving deep irradiation of pituitary
1½	22nd day	0 44	Vaginal bleeding, breast development Nipple pigmentation
6	9 2 51	0 58	Enlarged breasts and vaginal bleeding 2 years earlier. Monthly discharge since and onset of 'Petit Mal'
7	7. 8.52 11 8.52	0 4 0 6	Precocious menstruation and sexual precocity
3¾	26 9 52 30. 9.52	1 3 0 7	Advanced physical development, axillary and pubic hair, breast development, *no gonadotrophin* detected
6	31.10.52 1.11.52	0 7 0 7	Slight breast enlargement, advanced physical development

In all cases except one the values found were either within or below the range found for normal children of the same age. The first case, however, showed high values for both gonadotrophin and oestrone oestradiol, each of which was measured three times during two consecutive cycles. The child menstruated regularly, although only 2½. It

for this work. We are most grateful to Mr Stanley Clayton, Dr S. Leonard Simpson, Dr Raymond Greene and other clinicians whose patients we have investigated Thanks are also due to Messrs Organon who have generously supplied us with pure oestrogens.

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DISCUSSION

Dr Loraine: Did you use 4-point assays and how many animals were employed per dose level of standard and unknown preparation?

Miss Wood: Yes, we did 4-point assays. As a rule, fifteen animals were injected for each dose of standard and unknown, and twenty when we had enough material

Dr Loraine. I wonder if you have any information on the index of precision (λ) in these assays. Was that calculated?

Miss Wood: It was not calculated, but we estimated that there was an error of $\pm$ 30% at least.

Dr Klopper: I was intrigued by your very low values for pregnanediol excretion in normal cycles. You recorded several values of $<$ 1 mg/24 hr. What was the method you used?

Miss Wood: We used the method of Sommerville, Gough & Marrian, but we do not really consider that any value $<$ 1 mg/24 hr is accurate

Dr Klopper: If I remember rightly, Prof. Marrian put the limits of accuracy of his method at some 2 mg/24 hr.

My other point is: how did you estimate the substance you labelled 'FSH'? Did you use the Klinefelter method?

Miss Wood: We extracted our specimens by kaolin adsorption, and used the increase in uterine weight in infantile mice for assay.

Dr Klopper. Are you confident in labelling this material 'FSH'?

Miss Wood. No, we are not. We should have altered the labels on some of our slides I actually referred to it as 'gonadotrophin' in speaking We think it is FSH, but we would not like to commit ourselves.

Dr Boscott I was interested in your results on oestrone and oestradiol in cases of adrenal tumours, because Migeon & Gardner have recently shown by fluorimetric and chemical methods that in certain patients with adrenal tumours there is a raised urinary oestrogen output

Have you ever assayed oestrogens in human urines after treatment with ethinyl

Oestrone oestradiol excretion in other conditions

Single investigations were made in a number of other conditions, but as the amounts of oestrogen found fell within normal range, they were of little value. These include oligo- and hypomenorrhoea (three cases), dysmenorrhoea (two cases), Cushing's syndrome (two cases) and gynaecomastia (three cases).

DISCUSSION

The error involved in the biological assay of oestrogens makes the use of large numbers of animals advisable if accurate results are to be obtained; this in turn necessitates the collection of large volumes of urine, particularly from patients with a low oestrogen output, in order to obtain sufficient material for assay.

Estimation of urinary oestrogens at frequent intervals during normal menstrual cycles showed that it was possible to obtain consistent results using comparatively small numbers of mice, and our findings compared well with those of Pedersen-Bjergaard & Tønnesen [1948], Smith & Smith [1952] and other workers

It is clear from the study of oestrogen excretion in the conditions mentioned above that assay of oestrone oestradiol alone is seldom of much value. Simultaneous estimation of urinary gonadotrophins is essential when investigating cases of amenorrhoea, a persistently high output of gonadotrophin being in itself diagnostic of ovarian failure, and records of basal temperature or pregnanediol output are useful in detecting ovulation. In most cases of amenorrhoea only a series of investigations will reveal the true condition In cases of precocious puberty or in adult women where amenorrhoea is associated with hirsutism it is usual first to estimate the urinary neutral 17-ketosteroids, as these may be greatly increased when the condition is secondary to adrenocortical hyperplasia or tumour, and may eliminate the need for oestrogen assay. Oestrogen estimations should always be performed on more than one specimen from the same patient in order to take into account any cyclical or day-to-day variations, and it is preferable to have patients in hospital to ensure collection of complete urine specimens over periods of 2 or 3 days

It follows that biological oestrogen assays are of value in some conditions, but are to be avoided if conclusive results can be obtained by other means The preparation and assay of urine extracts is laborious and time consuming and the maintenance of a large colony of ovariectomized mice is expensive, in addition the experimental error, like that of most biological methods, is large

We have seen that considerable advances have recently been made in the determination of urinary oestrogens by chemical methods. The results we have obtained by a less accurate method indicate some of the problems which require reinvestigation using an improved technique.

It is probable that, particularly where such small quantities as those found in children and some cases of amenorrhoea are concerned, such methods will yield results of greater significance.

We wish to thank Prof C. H Gray for valuable advice and for providing the facilities

THE VALUE OF OESTROGEN DETERMINATIONS TO THE CLINICIAN

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The extent to which the clinician is likely to call for oestrogen determinations depends on many factors. An important one is his attitude towards the laboratory. There is, I think, a current tendency for the clinician to rack his brains to think up every conceivable laboratory and other investigation in order to 'work up' the case as completely as pos- [illegible] to think of the [illegible] y-staffed steroid [illegible]

Another factor is the degree of inconvenience to which the patient may be put. If one is investigating a case of gonadal or adrenocortical tumour—and such cases are, of course, excessively rare—the patient will be in hospital, 24 hr specimens of urine and frequent samples of blood can be easily and conveniently collected, and the laboratory staff will be as enthusiastic and excited as the clinician concerning the outcome of the investigations. But if, on the other hand, one is minded to study the urinary steroid excretion pattern in a woman suffering from non-ovulatory cycles or metropathia haemorrhagica, one is liable to encounter a certain amount of patient-resistance if one calls for frequent or serial 24 hr specimens. 'Over-night' specimens, however, are relatively convenient to collect, and a co-operative patient will usually consent to supply these.

Another factor which must be taken into account is how urinary (or blood) oestrogen determinations compare in complexity of technique, expense and informative value with other available methods of demonstrating oestrogen activity. Take, as an example, menstrual disorders. Oestrogen and oestrogen/progesterone activity may be studied by analysing basal temperature records, describing the histological findings of a specimen obtained by endometrial biopsy and evaluating the cornification index and other features of daily vaginal smears, as well as by determining the total oestrogen (and possibly the oestrone-oestradiol and oestriol ratios), and the pregnanediol output in the urine. In the first place it depends on what information is required If you want to decide whether ovulation is taking place, daily basal temperature records will provide a rough check and may even indicate not only that ovulation has occurred in any particular cycle, but also *when* it occured.

Endometrial biopsy inflicts upon the patient anything from mild discomfort to quite severe pain or even an anaesthetic and a night in hospital. Patients in this country may submit to it one or twice, but not repeatedly. Performed on the first day of menstrual bleeding it will provide information on whether progesterone has been present and whether the endometrium has responded satisfactorily to the oestrogen/progesterone

oestradiol? It is the most potent orally-active oestrogen in existence, but nothing is known of its metabolism. Could you throw any light on this subject?

Miss Wood: We have estimated oestrone oestradiol in urine of patients who were being treated with comparatively small quantities of ethinyl oestradiol, and, as far as we could tell, it made very little impression on the oestrogen output. But we did not do any proper quantitative recovery experiments. It disappeared; it was not being excreted in the urine.

Dr Boscott: Perhaps it is excreted in the faeces.

Dr Jellinck: I have done some experiments in which ethinyl oestradiol was injected into rabbits, and most of the activity seemed to be in the faeces. There was very little in the urine at all, and there was no evidence that it was converted into either oestrone or oestradiol.

Dr Diczfalusy: I wonder whether in your 4-point assays you have ever noticed a significant deviation from parallelism for your urinary extracts?

Miss Wood: No, we have not. We did quite a lot of preliminary work on this, but we found no significant deviation

Dr Diczfalusy: About this question of ethinyl oestradiol: Stimmel & May [in the J clin. Endocrin., 1951] claimed there is only ethinyl oestradiol in the urine after injection of 2 mg of this oestrogen, and that no oestrone, oestradiol or oestriol was found.

Miss Wood We have not really done enough work on this to be able to say anything.

adrenocortical tumours in males who may or may not show feminization, or in certain cases of gynaecomastia. Here the determination of the oestrogen is essential and complementary to the determination of the other hormone.

Ovarian tumours. Perhaps the most valuable use of urinary oestrogen determinations concerns the differentiation between ovarian tumours producing sexual precocity and the far more common condition of constitutional precocious puberty. Such ovarian tumours are usually granulosa cell tumours, though Seckel [1946] has described a case of chorio-epithelioma with a urinary oestrogen content equivalent to 600 i u. of oestrone per 24 hr and a high concentration of chorionic gonadotrophin Removal of the tumour resulted in disappearance of both C.G. and oestrogen from the urine. Granulosa cell tumours occurring in childhood are extremely rare, and Seckel was able to collect only thirty-one cases from the literature between 1926 and 1944. High blood and urinary oestrogen levels have been recorded in some of the recent cases, with absence of urinary gonadotrophins, whereas in constitutional (or neurogenic) precocious puberty oestrogen levels do not exceed the normal adult range and gonadotrophins are usually demonstrable.

Testicular tumours. Occasionally interstitial cell tumours which, when they occur in childhood, characteristically give rise to isosexual precocity, are associated with gynaecomastia. Of the seventeen cases of interstitial cell tumours occurring before puberty which I have been able to collect from the literature [Bishop, 1954], two developed gynaecomastia [Huffman, 1941; Jolly, 1954]. Other feminizing interstitial cell tumours have been described in adults [Monaschkin, 1926; Hunt & Budd, 1939; Melicow, Robinson, Ivers & Rainsford, 1949]. Unfortunately, oestrogen determinations were not undertaken in these cases, but it seems possible that excess of this hormone was the cause of the feminization, for Maddock, Epstein & Nelson [1952] found increased quantities of urinary oestrogen in two cases in which the only functional testicular structures were clumps of Leydig cells. They also treated men with normal testes with chorionic gonadotrophin, which [illegible] ization of the seminiferous tubules [illegible] There is, however, no general agreement that the Leydig cells are the source of testicular oestrogen as well as androgen. Huggins & Moulder [1945] described Sertoli cell tumours in five dogs with enlarged mammary glands and other signs of feminization. Two of the tumours yielded appreciable quantities of oestrogen. Teilum [1946] has published the only account of a Sertoli cell tumour in an otherwise normal man with descended testicles Unfortunately, oestrogen determinations were not undertaken in this case, but the patient complained of gynaecomastia and impotence. McCullagh & Schaffenburg [1952] suggest that the testicular oestrogen is elaborated by the germinal epithelium, possibly by the spermatids and spermatozoa, and the observations of Diczfalusy to-day are of great interest in this connection. It is clearly important to carry out oestrogen determinations wherever possible in these rare cases of testicular tumour and in all cases of gynaecomastia, to discover the source of testicular oestrogen and to define the different causes of gynaecomastia, which may result on the one hand from exogenous oestrogen administration and, on the other, from injection of chorionic gonadotrophin which induces physical signs of androgen production as well as gynaecomastia (or

influence; or whether progesterone has been deficient in its concentration or effect on the endometrium; or whether it has been completely absent, possibly with continuous, unopposed or even excessive influence of oestrogen The endometrial picture depends not only on the concentration of oestrogen and progesterone, but the responsiveness of the endometrium to these hormones.

A study of the cytology of vaginal smears is a somewhat time-consuming and exacting labour, if modern techniques are employed. Daily smears are essential in most cases They can be taken by the patient herself if she is moderately intelligent, highly cooperative and somewhat introspective, and if she is provided with a series of microscopic slides to which paper clips have been attached to prevent the smears rubbing against each other; a diamond to cut the date of each smear onto the appropriate slide, and a pipette of the right length to reach the posterior vaginal fornix—attached to the pipette is a rubber bulb to provide suction; and finally, a slide jar containing fixing fluid consisting of equal parts of ether and 95% alcohol. Analysis of the smears reflects the effect of oestrogen and progesterone on the vaginal epithelium, this effect varying not only with the concentration of these hormones but also with the responsiveness of the vaginal mucosa Endometrial biopsy and vaginal smear studies often do not indicate the same degree of oestrogenic or oestrogen/progesterone effect This means that, in these cases, the endometrium and vaginal mucosa are not equally responsive

Finally, there are the chemical and biological methods of estimating oestrogens (and pregnanediol) in urine which have been so fully described and discussed to-day. Generally speaking, they require expensive and delicate equipment, high maintenance costs for solvents and other materials, or for the feeding and care of animals, rather skilled technicians in relation to the salary scale on which one is forced to operate and, finally, exceptionally skilled and well-trained steroid chemists and biologists such as the ladies and gentlemen who have been addressing you to-day. These methods of estimating urinary oestrogen give some idea not only of the quantity, but also of the quality of the oestrogens which are being produced by the organism. but nevertheless they pin-point only one figure in a complex metabolic cycle which is dependent on such factors as rate of secretion and circulation, extent of conjugation, efficiency of uptake by the target tissue—which may involve enzyme systems and other complicated mechanisms—as well as renal clearance and the extent of recovery of hormone from the urine Like the various tests of thyroid function, the tests of oestrogenic activity reflect different aspects of the problem. An estimation of the serum protein-bound iodine gives an indication of the concentration of circulating thyroid hormone A determination of the radioactive iodine uptake by the thyroid gland shows how rapidly and efficiently the gland receives its raw material. Radioactive iodine clearance studies show how quickly the hormone circulates and is excreted, and an estimation of the basal metabolic rate indicates how the organism as a whole is responding to the thyroid hormone with which it is being supplied.

Yet another factor is the desirability of determining hormone ratios, such as the gonadotrophin/oestrogen ratio in order to locate the site of a lesion in the pituitary/ovarian axis; or the oestrogen/pregnanediol ratio in a case of menstrual disorder in order to evaluate the degree of corpus luteum failure, or the oestrogen/androgen ratio in certain

gaard & Tonnesen [1951] in which oestrogen and gonadotrophin assays were undertaken. Except in some cases of metropathia haemorrhagica in which they found excessive oestrogen output, all their other cases showed low oestrogen production. These results roughly coincide with those of de Allende & Orias [1950] who made a most detailed study of the vaginal smears of women with menstrual disorders and seldom encountered high oestrogen values in amenorrhoea, oligomenorrhoea or anovulatory cycles associated with menorrhagia, but did find high cornification indices in cases of metropathia haemorrhagica. Interesting as this information may be, I do not feel that we are likely to make use of routine oestrogen determinations in a gynaecological endocrine clinic. We shall, however, always have the odd patient whose steroid hormone pattern we shall be studying, and I think the results of such studies may prove eventually to be of value.

In conclusion, therefore, I would say that the methods which have been described to-day will not for some time become routine methods of clinical investigation, in the way that 17-ketosteroid, and possibly FSH determinations, have become popular with the clinician. Furthermore, I think it is important that the steroid chemist and biologist should be shielded from the uninformed enthusiasm of his clinical colleague as far as oestrogen determinations are concerned. On the other hand, I am delighted to find that there are now available reliable methods of providing the clinician with accurate quantitative and qualitative information concerning oestrogen output in specially selected cases. I believe this symposium has proved beyond doubt the need for endocrine laboratory units attached to clinics dealing exclusively with clinical endocrine material, and I am convinced that close co-operation between the clinic and the laboratory in such a set-up will lead to the clarification of many diagnostic problems and may encourage rational as opposed to empirical endocrine therapy, especially in menstrual disorders. While, therefore, I applaud the enterprise of the promoters of this symposium, I warn them against the indiscriminate enthusiasm of their clinical colleagues Oestrogen determinations are still, in my opinion, a research problem and not a routine laboratory demand.

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'puberty mastitis') in adolescent youths. Chorioepithelioma of the testis is usually associated with high oestrogen and C.G. excretion, as well as gynaecomastia, and other testicular tumours such as seminomas and mixed epitheliomas have been shown to yield high oestrogen values [Hamburger, 1938; Twombly & Hocker, 1941].

Adrenocortical tumours. Whereas the characteristic feature of these tumours is the high output of 17-ketosteroids and percentage of dehydroepiandrosterone, nineteen cases have been described as occurring in men (one was a child of 5) and giving rise to feminization, namely gynaecomastia, impotence, azoöspermia and testicular atrophy. In six of these cases oestrogen determinations have been undertaken and have been found to be uniformly high Diczfalusy & Luft [1952] submitted the urine in their case to countercurrent distribution and fluorimetric analysis—250 ml. of urine yielded 34.5 mg of free oestriol. After hydrolysis 68.5 mg of oestrone, 18.4 mg of oestradiol and 857 mg of oestriol were recovered from the same specimen of urine. Unfortunately, in only three of these six cases were 17-ketosteroid studies also undertaken. In one of these, reported by Luft & Sjögren [1949], the 17-ketosteroid output was disproportionately low compared with the yield of oestrogens. It is clearly of great importance that combined studies of 17-ketosteroids and oestrogens should be carried out in future cases of this very rare condition.

Pregnancy disorders. The intriguing theory of the Smiths [1948] that many disorders of pregnancy, such as certain cases of habitual and threatened abortion, pregnancy vomiting, toxaemia, pre-eclampsia, eclampsia and diabetes are associated with an imbalance of placental hormone production, leading to excessive output of chorionic gonadotrophin and diminished quantities of urinary oestrogen and pregnanediol, and that this imbalance may be readjusted by administration of oestrogen (especially stilboestrol) and possibly progesterone, has stimulated the imagination of obstetricians and diabetic experts as well as steroid chemists, and is still the subject of serious, though essentially benign controversy. The modern techniques of oestrogen determination which have been laid before us to-day must obviously be applied to this problem, and this is one of the very few conditions in which blood, as opposed to urinary, oestrogen estimation may prove valuable According to Borth & de Watteville [1952] 1.6 to 3 i.u. of oestrone/ml of serum is the normal range in the last trimester Apart from the Smiths' work there are other fascinating problems which confront the obstetrician and his endocrine laboratory. One is the sudden fall in oestrogen output in cases of missed abortion and foetal death.

Menstrual disturbances So far as I can see, the study of hormonal patterns in menstrual disorders is an almost uncharted sea. Some of the pioneer pilots of those uncharted oceans are here to-day It is obvious to any clinician who has prescribed hormone therapy 'in blinkers' for menstrual disorders as long as I have, that no further progress can be made without an endocrine laboratory unit There are few who have so far had such facilities De Watteville, in my opinion, is the outstanding example, and for the results of his experience I refer you to his recent article [Borth & de Watteville, 1952]. In this he adopts a rather conservative attitude to the clinical value of the work which he reviews. He refers to the studies of 553 cases of menstrual disorder by Pedersen-Bjer-

oestrogen in semen comes from the testis, but is rather a biological curiosity, and probably has no physiological function.

That brings me to Dr Mitchell's paper on the oestrogen content of the placenta. Here again, the chief query raised by this paper to biologists is what the oestrogen is doing in the placenta, and whether it has any physiological function. The first difficulty in dealing with this particular question is the fact that different species vary so much in the oestrogen content of the placenta, and it is difficult to visualize that it can have any function generally applicable to mammals. I think I am right in saying that, with the definite exception of the cow and the possible exception of the horse, oestrogen is not known to be abundant in the placenta of mammals other than man. That raises the next and rather obvious comment that the endocrinological characteristics of the various reproductive processes appear to vary more from species to species than any of the other endocrinological functions. As I was listening this afternoon, I recalled the remarkable endocrinological differences between the pregnant mare and the pregnant woman. The former has a very large amount of gonadotrophin of the 'pregnant mare's serum' type circulating in the blood, but not much oestrogen which, however, appears in quantity in the urine. By contrast, in women, there is some gonadotrophin and some oestrogen circulating in the blood, and early on a large amount of gonadotrophin and later a considerable amount of oestrogen in the urine. The type of gonadotrophin is entirely different from that in the mare

When the next paper was read by Miss Wood, I began to feel entirely at home, the word 'biological assay' was mentioned, and the various difficulties were emphasized. One of the reasons it is so satisfactory to see chemical superseding biological methods of oestrogen determination is that there are so many different factors which cause variation in the result of biological assay—a slightly different method of comparing the unknown with the standard will produce very different results I well remember that my first contact with our Chairman, Sir Charles Dodds, arose many years ago because we were obtaining different results in the biological assay of the same preparations of oestrogens. We had begun to have grave suspicions of each other when it turned out that Prof. Dodds' team were using six injections on rats, while we were using four injections on mice, a serious discrepancy was thus explained

There is another factor in biological assay which makes things very difficult, and that is the degree of purification of the preparation It is one of the anomalies of biological assay that the more purified the preparation, the less active it appears in many of the ordinary tests. That, of course, is because the purified substance is absorbed very rapidly and excreted without having time to do its job Many of those undertaking biological assays for a chemist have got into trouble, because the better he worked the less they made of his results Having said this about biological assay, I should add that I realize full well that those engaged in chemical assays also have their troubles, and I was interested [illegible]

labour for everybody concerned, and it is obviously not quite certain in his mind, and

GENERAL DISCUSSION

(AFTERNOON SESSION)

Dr Parkes: I have been out of active participation in this particular subject for a number of years, and I am not as well up in the details as I ought to be to open this general discussion. There are, however, a number of general remarks which I think can usefully be made about the conference. The first relates to the interesting division of the two sessions and various papers between chemical and biological techniques. In the past, biological methods of assay had to be relied upon almost entirely. By contrast, in planning today's symposium the original idea was to devote the morning session to chemical techniques and the afternoon to biological techniques. In practice the chemical techniques have overflowed into the afternoon session, and in only one paper have the authors *depended entirely on biological* assays. This, *I think*, is a very definite sign of progress. In 1938 there was a long and arduous international conference on the establishment of international standards for the pituitary hormones concerned with reproduction. At the end of this conference we issued a report, which recorded a remark made by Sir Henry Dale that the ultimate aim of biological assay was self-abolition. I think, today has shown that this highly desirable process has gone a long way. This conclusion seems to be backed up by the fact that if participation in the discussions is any guide, then the members of this audience are more chemically than biologically minded.*

Turning to the papers of this afternoon's sessions, there is first the interesting paper by Dr Diczfalusy on the oestrogen content of human semen. I have some particular interest in this paper because years ago, following persistent reports of small amounts of oestrogen in semen, I was lured into doing some biological experiments, and after some little work obtained results that were rather inconclusive. I was very glad to hear today that the definite answer was obtained, on what appears to have been abundant material, that oestrogens (all three of the common oestrogens, apparently) undoubtedly occur in human semen. That is an interesting observation. But the biologist immediately asks, 'Where does the stuff come from? And what does it do there?' The answer to the first question seems pretty clear, and has already been elaborated by Dr Bishop. The oestrogen presumably comes from the testis, probably from the seminiferous tubules, and may be closely associated with the spermatozoon. The question of what, if anything, is its physiological function in semen is, of course, much more difficult to answer. There have been various suggestions from time to time, but I think we must remember that the amounts present, in terms of biological reactions, are very small indeed. Half a gallon of human semen is not a physiological amount. The amount of oestrogen available from physiological quantities of semen, although it might perhaps do something to a rat, is not likely to produce an effect of any kind in its natural habitat. I suggest, therefore, that the

*In view of Sir Charles Dodds's comments later in closing the discussion I ought to add that in making these remarks I had in mind only the assay of known oestrogens or oestrogenic preparations. Biological testing is, of course, usually unavoidable in the first qualitative examination of new compounds or preparations for oestrogenic activity.

methods of estimation when we know we are expecting rather larger differences than we think. A simplification at the expense of accuracy may be quite permissible when we are looking for large differences. But I think in the end—and Dr Swyer made this point in our paper this morning—only the blood values will have any meaning at all, or the blood together with urinary values.

Dr Parkes: I think it is quite evident that the information wanted is as to what is going round in the circulation, not what is being excreted. Until what appears in the urine is shown to have some significant relation to what is going round inside, I think the whole position is not on secure ground.

Miss Stern: In defence of our method of doing oestrogen determinations on urine, we have started it because urine was the easiest fluid to get hold of and without much bother to the patient; with blood we may need quite large amounts. Also, one of the ultimate aims after examining oestrogen in blood is oestrogen metabolism, and before we can do that, we must have accurate methods for determining oestrogens in urine.

Dr Brown While admitting that blood oestrogen methods are very desirable, I would like to put in a word for urinary oestrogen estimations. In all the menstrual cycles that we have been studying, the features have been so constant that we were able to predict, without any reference to basal temperature curves etc., where ovulation occurred, when menstruation occurred, and so on. I think for the first time in history we are able to determine what the ovary is doing without having to rely on menstruation or any of its outward physical signs, and that seems very valuable in itself.

Dr Parkes: That is an instance of the type I mentioned, where you are able to show that what you measured in the urine did give an indication of what was going on inside. That is what we want.

Dr Rumney. I do not want to provoke Dr Parkes any further in connection with the oestrogen production by the stallion This may be a curiosity, but I think it is established that the adrenal cortex produces, in addition to corticosteroids, oestrogens and androgens, while I think the late Dr Dingemanse showed that both androgens and oestrogens are produced by the ovary. Again, virilization in the Stein-Leventhal syndrome is believed to be due to ovarian androgens I do not see why, if the ovary can produce both oestrogens and androgens, the testis should not normally produce both these substances as well.

Dr Parkes: There is no reason at all—in fact it does. May I make one more comment about this stallion business. Shortly after Zondek made that remarkable observation of the enormous concentration of oestrogen in the stallion urine, it became popular to suppose that the ordinary benign enlargement of the prostate in man had some connection with the oestrogen story In association with Prof. Zuckerman, we went to some trouble to get the prostates of a number of stallions, with particular reference to the uterus masculinus which is quite a well-defined organ in the horse The point was that injected oestrogen, irrespective of what it may do to the prostate, undoubtedly causes changes in the uterus masculinus of experimental animals. Yet in the stallion we could find no changes whatever in the uterus masculinus suggesting that oestrogen had been acting on it. And yet the animal excretes 100,000 units a day.

Dr Swyer An almost analogous situation arises in the case of the pregnant woman given

it has never been quite certain in mine, whether the amount of information given about the patient—I am not talking about academic or chemical or biological matters—really justifies it. That is undoubtedly one of the things that will be cleared up in future. Part of this difficulty seems to be due to the fact that over the years there has been this intense concentration on what appears in the urine which, for all we know, may be merely what the organism does not want, and which may therefore not give much indication of what is going on inside the organism. Years ago I mooted the idea that it would be a good thing to get away from urine and back to blood, and I think I am still justified in repeating that remark. But my chief function here is to provoke other people into speaking, and I hope I have at least done that.

Dr Diczfalusy: I feel I must protest against Dr Parkes' conclusions about the oestrogen secretion of the testes He considered it in the nature of a biological curiosity that there is oestrogen in human semen, and that one simply cannot understand why it should be there. Now, I understand that it was a provocative remark because Dr Parkes wanted us to form an opinion on this problem. I do not see why one might not assume that the testis, as an organ of internal secretion, would secrete oestrogens, and that these oestrogens probably have an important function in acting on pituitary gonadotrophin secretion. I suppose that was the reason why people looked so intensely for the existence of a second testicular hormone.

Dr Parkes If, as I take it, you refer to the assumed oestrogenic secretion of the testis, that may or may not have some function to perform. What I meant to say was that it was not in the least evident to me what physiological function was performed by such part of that secretion as got into the semen.

Dr Diczfalusy I misunderstood.

Dr Boscott. I wonder if Dr Parkes would like to comment on another species difference He compared the pregnant mare with the human female How about the stallion compared with the human male? The stallion excretes as much as 20 mg of oestrone/litre, whereas the human male excretes very much less than that

Dr Parkes I regard the stallion as very much to the point in showing that these biochemical curiosities of secretion or excretion do occur and are very difficult to explain in any rational manner, and indeed may have no rational explanation at all The fact that the stallion excretes in its urine 100,000 units a day—or something similarly enormous—is just one of those things I do not think it means anything in particular

Dr Hannelore Braunsberg I should very much like to support Dr Parkes' 'back-to-blood' movement We are all busy studying the excretion of oestrogens, and there are certain aspects which might be worth considering in that connection, such as the renal function, which seems to be ignored If you consider the presence of free oestrogens as well as conjugates, surely the degree and efficiency of renal function must bear some relation to the amount excreted, and that might obscure any physiological effects Again, the day-to-day and hourly variations may do so, and even daily variations may obscure any physiological differences by our not doing enough daily specimens In connection with the clinical value of any oestrogen excretion values, surely the difference between normal and abnormal individuals is not yet established, and we may be able to simplify our

MEMOIRS OF THE SOCIETY FOR ENDOCRINOLOGY

No 1. The Thyroid Gland, July 1953

No. 2. The Determination of Adrenocortical Steroids and their Metabolites, November 1953

No 3. The Technique and Significance of Oestrogen Determinations, February 1955

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